WAGONER, KAIRA MALINDA, Ph.D. An Investigation of the Relationships Between Common Stressors, Brood-Signaling, Hygienic Behavior, and Selective Breeding in the Honey Bee (*Apis mellifera*). (2015)
Directed by Dr. Olav Rueppell. 165 pp.

Despite the importance of the honey bee (*Apis mellifera*) to scientific advancement, food security and natural ecosystems, managed honey bee colonies are dying at alarming rates in much of the Northern Hemisphere. Recent declines are largely attributed to anthropogenic stressors and to the spread of natural parasites and diseases, most notably the ectoparasitic mite *Varroa* (*Varroa destructor*), which is considered by many to be the most important threat to apiculture today. *Varroa* resistance programs have been successful and rely primarily on selection for hygienic behavior. However adequate mite resistance through hygienic behavior has not yet been fully achieved, and the signal responsible for triggering the hygienic removal of *Varroa*-infested brood has remained elusive. Employing behavioral, chemical and molecular analyses, the following dissertation investigates the relationships between honey bee stressors, chemical brood signals, and hygienic behavior for bees originating from three distinct breeding programs. Cross-fostering experiments and chemical analyses suggest that hygienic behavior is influenced by a specific chemical originating from honey bee brood, and that the stressor that triggers this chemical signal is different for brood originating from distinct breeding programs. Additional behavioral and chemical analyses provide evidence of increased iron content and higher rates of hygienic removal of brood from cells overlapping steel wires commonly used by beekeepers to add stability to wax-foundation frames. Improved understanding of the relationships between honey bee stressors, brood signals, and

hygienic behavior described in this dissertation has the potential to make a positive impact on the health of honey bees. The broad-scale applicability of results presented here stems primarily from the practicality of the solutions these results imply. Through the development of sustainable strategies to combat the *Varroa* mite, and by discouraging the use of steel wire stabilizers in wax-foundation frames, this work has the potential to improve honey bee health, and thus positively influence the honey bee's enormous contribution to the economy and the environment.

Keywords: Honey bee, hygienic behavior, brood-signaling, selective breeding, *Varroa*, wax foundation

AN INVESTIGATION OF THE RELATIONSHIPS BETWEEN COMMON STRESSORS, BROOD-SIGNALING, HYGIENIC BEHAVIOR, AND SELECTIVE BREEDING IN THE HONEY BEE (*APIS MELLIFERA*)

by

Kaira Malinda Wagoner

A Dissertation Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Greensboro
2015

Approved by

_____
Committee Chair

APPROVAL PAGE

This dissertation written by Kaira Malinda Wagoner has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

_____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEDGEMENTS

I would like to thank my advisor, Olav Rueppell, for his tremendous guidance and support in the completion of this research, from study design to data analysis to written and oral presentation of the final product. I would also like to thank my committee members, Marla Spivak, Malcolm Schug and Nadja Cech, for their guidance and support over that last four years. Thank you to Jeff Hull, Amy Weeks, Bob Danka and John Pletcher for supplying me with the bees needed for this research, and to Abraham Hefetz for his help with GC-MS peak identification. Thank you to the UNCG Biology Department, Project Apis m and the USDA for the funding that made this work possible. Thank you to Dominick Defelice, Carlos Vega Melendez, Babak Yousefi, and Reynaldo Diaz for help in the bee yard (sorry about the stings), and to Fran Shoemaker, Humberto Boncristiani, Daniel Smith, Quinton Irby, and Samantha Mcpherson for their hard work in the lab. Thank you to Bertrand Fouks and other members of the UNCG Social Insect Lab for your accolades, criticisms, and company. Last but not least, thank you to all of the honey bees that gave their lives for this research, and to those that live on, unknowingly enriching our lives, from flower to farmer to fork.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION AND BACKGROUND

Introduction

The complex nature of the honey bee *Apis mellifera* has long fascinated the keen observer. Those whose curiosity of bees prevails over their fear of the sting find themselves intrigued by the precise and systematic performance of numerous age-specific tasks within the colony. So intrigued was the famous entomologist Karl von Frisch that, in reference to findings from his own honey bee experiments, he declared, "no competent scientist *ought* to believe these things on first hearing." As Frisch went on to describe, the three unique honey bee castes use visual, chemical and tactile communication to achieve coherent, systematic organization within the colony (Frisch, 1950). This complexity, besides revealing the honey bee's intrinsic value and explaining the beekeeper's relentless fascination, renders the honey bee valuable as a model for behavioral studies of sociality, collaboration, navigation, learning, memory and longevity (Menzel, 2012). In addition to its importance as a behavioral model, the honey bee serves as an important model for molecular research, especially since the sequencing of the honey bee genome in 2006 (Weinstock et al., 2006). The honey bee is particularly pertinent to the advancement of human genomics considering that, compared to other sequenced insects, honey bee genomes are more similar to those of vertebrates in genes controlling

processes like DNA methylation, RNA interference, and circadian rhythm (Weinstock et al., 2006).

However the importance of the honey bee extends beyond the laboratory, into the field of agricultural science. Honey bees have an estimated $16.4 billion dollar effect on annual crop yield in the United States alone (Losey & Vaughan, 2006; Morse & Calderone, 2000) and globally, honey bees are the most important pollinator of large-scale agricultural crops (Klein et al., 2007). Furthermore, in light of recent declines in native pollinators (Biesmeijer et al., 2006; Grixti et al., 2009; Potts et al., 2010), honey bees have an increasingly important pollination function in natural ecosystems, and therefore contribute significantly to environmental health (Memmott et al., 2004; Potts et al., 2010). Humans have burdened the honey bee with the spread of natural parasites and diseases, direct exposure to known toxins, and the destruction of natural habitat. By improving our understanding of the honey bee, we have the potential to reduce both the frequency and severity of these threats. Doing so is of utmost importance as, with the honey bee's global significance to scientific advancement, food security, and natural ecosystems, a threat to honey bees can be considered a threat to humanity.

Background

Healthy honey bee colonies consist of drone, queen, and worker castes. Drones, the only male caste, originate from haploid eggs and have the sole function of mating queens from other colonies. Both queens and workers originate from diploid eggs, and are differentiated during early development by diet, cell size and cell orientation (Shi et

al., 2011; Winston, 1991). A young queen generally takes between 1 and 5 mating flights, during which she mates with several (up to 44) males (Hayworth et al., 2009). Sperm is stored in the queen's spermatheca for the duration of her reproductive life (Harbo, 1979). Honey bee colonies generally contain only one queen, who serves as the primary reproductive member and is capable of laying several thousand eggs per day (Winston, 1991). Each of the queen's eggs is laid in an individual hexagonal cell made of wax. In larger cells, the queen deposits unfertilized eggs that develop into drones. In smaller cells, the queen deposits fertilized eggs that can develop into either queens or workers. Healthy queens typically concentrate brood at the colony center where they are most protected from extreme temperatures. Approximately three days after being laid, eggs hatch into larvae, which are cared for by young nurse bees for another five to six days. At this time the bottom of the cell is filled with brood food, and the cell is capped over with a porous mix of wax and silk (Jay, 1964; Langstroth, 1914). Between 8-14 days after capping (depending on caste) the developed bee emerges from the cell as an adult.

Within the colony, the queen is continuously attended by workers, whose ovarian development is suppressed in the presence of queen mandibular pheromones (Hoover et al., 2003). Workers perform many different tasks as they age including (in chronological order) brood care, nectar receiving, pollen packing, comb building, colony ventilation, colony defense, and foraging. Allocation and initiation of tasks is based on age (Huang & Robinson, 1996; Winston, 1991), pheromone communication (Huang & Robinson, 1992), and genotypic differences (Page et al., 1995; Rueppell et al., 2004). Evolution of seemingly altruistic acts of workers such as reproductive restraint, food sharing, colony

defense and self-removal by diseased individuals (Rueppell et al., 2010) is rooted in parental care where non-dispersal or aggregations of same-aged offspring led to increased kin survival through division of labor between overlapping generations (Andersson, 1984; Bourke, 1995). Kin selection is also thought to have been facilitated by haplodiploidy, which results in a higher coefficient of relatedness between sisters of the same patriline (0.75) than would exist between workers and their own offspring (0.50) (Andersson, 1984; Crozier, 1970; Hamilton, 1964; Hughes et al., 2008). Though honey bee polyandry and queen-worker sex-ratio conflicts have led some to question the contribution of haplodiploidy to the evolution of sociality (Andersson, 1984), evidence of monogamy in ancestors of several independent lineages of eusocial bees confirm its importance (Hughes et al., 2008).

The high coefficient of relatedness and close contact between honey bees within a colony make honey bees, like other socials insects, highly susceptible to the horizontal spread of infectious diseases (Cremer et al., 2007; Evans & Schwarz, 2011; Möckel et al., 2011). Social activities such as food sharing, cannibalism of diseased brood, and contact with diseased brood during their removal from the hive (Cremer et al., 2007; Evans & Schwarz, 2011; Möckel et al., 2011) often lead to the spread of disease between honey bees. The exchange of parasites within the hive also facilitates the spread of honey bee diseases (Evans & Schwarz, 2011), as parasites like the ectoparasitic mite *Varroa destructor* (*Varroa*) are known to vector numerous honey bee pathogens (Bowen-Walker et al., 1999; Chen et al., 2004; Kanbar & Engels, 2003; Martin et al., 2012) and have been associated with both viral amplification and an increase in honey bee disease

susceptibility (Martin et al., 2012; Yang & Cox-Foster, 2005). Like pathogens, parasites can be spread between individual honey bees through close contact within the colony, use of shared foraging sites, and the movement of honey bees from one colony to another during drifting or robbing events (Cremer et al., 2007; Evans & Schwarz, 2011). The rapid spread of disease between honey bees is especially problematic considering that honey bees have relatively few genes associated with immunity, possessing only one-third of the immune-related genes of non-social insects like mosquitoes and flies (Evans et al., 2006).

The combination of natural disease susceptibility and the anthropogenic spread (Anderson & Trueman, 2000; Wenner & Bushing, 1996) of honey bee parasites and pathogens have been detrimental to honey bee health (Amdam et al., 2004; De Jong et al., 1982; Garedew et al., 2004; Gisder et al., 2009; Highfield et al., 2009; Nazzi et al., 2012; Schatton-Gadelmayer & Engel, 1988). However natural stressors are not acting alone, but in combination with several other anthropogenic stressors, such as exposure of honey bees to harmful agrochemicals (Henry et al., 2012; Johansen & Kleinschmidt, 1972; Potts et al., 2010; Stefanidou et al., 2003), to miticides used to control *Varroa* (Collins et al., 2004; Haarmann et al., 2002; Mullin et al., 2010; Pettis et al., 2004; Sylvester et al., 1999), and to poor nutrition as a result of the decline in diversity and availability of natural foraging habitat (Brodschneider & Crailsheim, 2010; Naug, 2009; Neumann & Carreck, 2010). Honey bees' deficit of genes encoding for detoxification enzymes like cytochrome P450 monooxygenases, carboxyl/ cholinesterase's, and glutathione-S-transferases further increase their sensitivity to chemical contaminants (Claudianos et al.,

2006). Making matters worse, immuno-suppression caused by chemical exposure makes honey bees more susceptible to parasites like *Varroa,* as well as to the pathogens they vector (Boncristiani et al., 2012; Ellis, 2012; Mullin et al., 2010; Spivak et al., 2011; vanEnglesdorp et al., 2008).

The synergy between (Johnson et al., 2009; Nazzi et al., 2012) and accumulation of multiple honey bee stressors has led to an inability of beekeepers to maintain sufficient numbers of colonies for the increasing global demand for crop pollinators (Aizen et al., 2008; Aizen & Harder, 2009; Holden, 2006; Kearns et al., 1998). Following pollinator population trends in much of the Northern Hemisphere (Genersch et al., 2010; Meixner, 2010; Potts et al., 2010), managed honey bee colonies in the United States have declined more than 50% over the last six decades, from around 5.9 million colonies in 1947 to around 2.4 million colonies in 2005 (Meixner, 2010; Pettis & Delaplane, 2010). In four of the last five years, total annual colony losses in the United States have exceeded 33%, and each year overwintering loss rates have been higher than levels deemed acceptable by beekeepers (Lee et al., 2015; Spleen et al., 2013; Steinhauer et al., 2015; Steinhauer et al., 2014; vanEngelsdorp et al., 2012).

While exposure to anthropogenic chemicals is a relatively recent threat to honey bee health, eusociality evolved in honey bees over 60 million years ago (Kapheim et al., 2015). As a result, honey bees have developed several mechanisms of social immunity for defense against the parasites and pathogens that spread so rapidly within communal living spaces. Examples of honey bee social immune mechanisms include induction of brood comb fever (Starks et al., 2000), entombment and hive construction using the

antimicrobial substance propolis (Evans et al., 2009; Kujumgiev et al., 1999; Simone et al., 2009), self-removal of unhealthy individuals (Rueppell et al., 2010), auto- and allo-grooming (Bozic & Valentincic, 1995; Waddington & Rothenbuhler, 1976), and hygienic behavior (Peng et al., 1987; Rath & Drescher, 1990; Rosenkranz et al., 1993; Spivak, 1996), the detection, uncapping and removal of diseased brood from the hive (Spivak & Reuter, 2001a). Hygienic behavior is an especially important trait as it targets *Varroa* mites which require honey bee brood to complete their reproductive cycle, and are considered to be the most serious threat to honey bee health today (Anderson & Trueman, 2000; Rosenkranz et al., 2010). Additionally, honey bees are highly vulnerable to stress during development, when rapid growth and metabolism occur (Winston, 1991).

One ability essential to successful performance of coordinated social immune mechanisms such as hygienic behavior is communication. As was first revealed by Karl von Frisch over 60 years ago, honey bee castes use visual, chemical and tactile communication to coordinate the execution of collaborative tasks within the colony (Frisch, 1950). General studies of animal behavior have made it clear that both the quality of the signal sent, and the sensitivity of the signal recipient are of great importance to successful communication (Endler, 1993). However, much of the research into chemical communication between honey bee brood and adults, such as that required for hygienic behavior, has focused on olfactory sensitivity of the adult, or signal recipient (Aumeier & Rosenkranz, 2001; Goode et al., 2006; Harbo & Harris, 2009; Ibrahim & Spivak, 2006; Masterman et al., 2001; Masterman et al., 2000; Spivak et al., 2003), rather than the brood, or origin of the signal.

The following dissertation describes the investigation of known biotic, and previously unresolved abiotic honey bee stressors, testing the hypotheses that 1) selection for hygienic behavior in honey bees has influenced brood signaling, 2) hygienic behavior and related stressors are associated with chemical brood signals specific to honey bee brood type, and 3) steel wires commonly used to add stability to wax comb foundation negatively affect honey bee brood health. Hypotheses 1, 2, and 3 as listed above are discussed in chapters II, III, and IV, respectively. Findings from this research facilitate the improvement of honey bee health by linking honey bee stressors, brood signals, and hygienic behavior (Chapters II and III), and thus by providing tools for the development of novel strategies to combat the *Varroa* mite and the pathogens it vectors. Findings from this research also facilitate the improvement of honey bee health by revealing the harmfulness of a practice common to modern beekeeping (Chapter IV). The focus of this work on *sustainable* solutions is a response to the severity of recent honey bee health threats, the improbability of reversing the spread of harmful honey bee parasites and pathogens, and the numerous anthropogenic burdens that continue to be placed on honey bees, sometimes in an effort to help. Though the topic of this work is highly focused, its potential impact is broad, as the honey bee's importance spans beyond the economic and environmental interests of the beekeeper, scientist, farmer and environmentalist, to a multitude of intrinsically valuable plant and animal species.

References

Aizen MA, Garibaldi LA, Cunningham SA & Klein AM (2008) Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. Current Biology 18: 1572-1575.

Aizen MA & Harder LD (2009) The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Current Biology 19: 915-918.

Amdam GV, Hartfelder K, Norberg K, Hagen A & Omholt SW (2004) Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite *Varroa destructor* (Acari: Varroidae): a factor in colony loss during overwintering? Journal of Economic Entomology 97: 741-747.

Anderson D & Trueman J (2000) *Varroa jacobsoni* (Acari: Varroidae) is more than one species. Experimental and Applied Acarology 24: 165-189.

Andersson M (1984) The Evolution of Eusociality. Annual Review of Ecology and Systematics: 165-189.

Aumeier P & Rosenkranz P (2001) Scent or movement of *Varroa destructor* mites does not elicit hygienic behaviour by Africanized and Carniolan honey bees. Apidologie 32: 253-264.

Biesmeijer JC, Roberts S, Reemer M, Ohlemüller R, Edwards M, Peeters T, Schaffers A, Potts S, Kleukers R & Thomas C (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313: 351-354.

Boncristiani H, Underwood R, Schwarz R, Evans JD & Pettis J (2012) Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. Journal of Insect Physiology 58: 613-620.

Bourke AF (1995) Social Evolution in Ants. Princeton University Press.

Bowen-Walker P, Martin S & Gunn A (1999) The Transmission of Deformed Wing Virus between Honeybees (*Apis mellifera* L.) by the Ectoparasitic Mite *Varroa jacobsoni* Oud. Journal of Invertebrate Pathology 73: 101-106.

Bozic J & Valentincic T (1995) Quantitative analysis of social grooming behavior of the honey bee *Apis mellifera carnica*. Apidologie 26: 141-141.

Brodschneider R & Crailsheim K (2010) Nutrition and health in honey bees. Apidologie 41: 278-294.

Chen Y, Pettis JS, Evans JD, Kramer M & Feldlaufer MF (2004) Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. Apidologie 35: 441-448.

Claudianos C, Ranson H, Johnson R, Biswas S, Schuler M, Berenbaum M, Feyereisen R & Oakeshott J (2006) A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Molecular Biology 15: 615-636.

Collins AM, Pettis JS, Wilbanks R & Feldlaufer MF (2004) Performance of honey bee (*Apis mellifera*) queens reared in beeswax cells impregnated with coumaphos. Journal of Apicultural Research 43: 128-134.

Cremer S, Armitage SAO & Schmid-Hempel P (2007) Social Immunity. Current Biology 17: R693-R702.

Crozier RH (1970) Coefficients of relationship and the identity of genes by descent in the Hymenoptera. American Naturalist: 216-217.

De Jong D, De Jong P & Goncalves L (1982) Weight loss and other damage to developing worker honeybees from infestation with *Varroa jacobsoni*. Journal of Apicultural Research 21: 165-167.

Ellis J (2012) The honey bee crisis. Outlooks on Pest Management 23: 35-40.

Endler JA (1993) Some general comments on the evolution and design of animal communication systems. Philosophical Transactions of the Royal Society B: Biological Sciences 340: 215-225.

Evans J, Aronstein K, Chen Y, Hetru C, Imler JL, Jiang H, Kanost M, Thompson G, Zou Z & Hultmark D (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect Molecular Biology 15: 645-656.

Evans JD, Donovall L, Mullin C, Frazier M, Frazier J, Tarpy DR, Hayes J & Pettis JS (2009) "Entombed Pollen": A new condition in honey bee colonies associated with increased risk of colony mortality. Journal of Invertebrate Pathology 101: 147-149.

Evans JD & Schwarz RS (2011) Bees brought to their knees: microbes affecting honey bee health. Trends in Microbiology 19: 614-620.

Frisch Kv (1950) Bees: Their Vision, Chemical Senses, and Language. Cornell University Press, Ithaca, NY.

Garedew A, Schmolz E & Lamprecht I (2004) The energy and nutritional demand of the parasitic life of the mite *Varroa destructor*. Apidologie 35: 419-430.

Genersch E, Von Der Ohe W, Kaatz H, Schroeder A, Otten C, Büchler R, Berg S, Ritter W, Mühlen W & Gisder S (2010) The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41: 332-352.

Gisder S, Aumeier P & Genersch E (2009) Deformed wing virus: replication and viral load in mites (*Varroa destructor*). Journal of General Virology 90: 463-467.

Goode K, Huber Z, Mesce KA & Spivak M (2006) Hygienic behavior of the honey bee (*Apis mellifera*) is independent of sucrose responsiveness and foraging ontogeny. Hormones and Behavior 49: 391-397.

Grixti JC, Wong LT, Cameron SA & Favret C (2009) Decline of bumble bees (Bombus) in the North American Midwest. Biological Conservation 142: 75-84.

Haarmann T, Spivak M, Weaver D, Weaver B & Glenn T (2002) Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. Journal of Economic Entomology 95: 28-35.

Hamilton WD (1964) The genetical evolution of social behaviour. Journal of Theoretical Biology 7: 17-52.

Harbo J (1979) The rate of depletion of spermatozoa in the queen honeybee spermatheca. Journal of Apicultural Research 18: 204-207.

Harbo JR & Harris JW (2009) Responses to *Varroa* by honey bees with different levels of *Varroa* Sensitive Hygiene. Journal of Apicultural Research 48: 156.

Hayworth MK, Johnson NG, Wilhelm ME, Gove RP, Metheny JD & Rueppell O (2009) Added weights lead to reduced flight behavior and mating success in polyandrous honey bee queens (*Apis mellifera*). Ethology 115: 698-706.

Henry M, Beguin M, Requier F, Rollin O, Odoux J-F, Aupinel P, Aptel J, Tchamitchian S & Decourtye A (2012) A common pesticide decreases foraging success and survival in honey bees. Science 336: 348-350.

Highfield AC, El Nagar A, Mackinder LC, Laure M-LN, Hall MJ, Martin SJ & Schroeder DC (2009) Deformed wing virus implicated in overwintering honeybee colony losses. Applied and Environmental Microbiology 75: 7212-7220.

Holden C (2006) Report warns of looming pollination crisis in North America. Science 314: 397-397.

Hoover SER, Keeling CI, Winston ML & Slessor KN (2003) The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften 90: 477-480.

Huang Z-Y & Robinson GE (1992) Honeybee colony integration: worker-worker interactions mediate hormonally regulated plasticity in division of labor. Proceedings of the National Academy of Sciences 89: 11726-11729.

Huang Z-Y & Robinson GE (1996) Regulation of honey bee division of labor by colony age demography. Behavioral Ecology and Sociobiology 39: 147-158.

Hughes WOH, Oldroyd BP, Beekman M & Ratnieks FL (2008) Ancestral monogamy shows kin selection is key to the evolution of eusociality. Science 320: 1213-1216.

Ibrahim A & Spivak M (2006) The relationship between hygienic behavior and suppression of mite reproduction as honey bee (*Apis mellifera*) mechanisms of resistance to *Varroa destructor*. Apidologie 37: 31.

Jay S (1964) The Cocoon of the Honey Bee, *Apis mellifera* L. The Canadian Entomologist 96: 784-792.

Johansen CA & Kleinschmidt MG (1972) Insecticide formulations and their toxicity to honeybees. Journal of Apicultural Research 11: 59-62.

Johnson RM, Pollock HS & Berenbaum MR (2009) Synergistic interactions between in-hive miticides in *Apis mellifera*. Journal of Economic Entomology 102: 474-479.

Kanbar G & Engels W (2003) Ultrastructure and bacterial infection of wounds in honey bee (*Apis mellifera*) pupae punctured by *Varroa* mites. Parasitology Research 90: 349-354.

Kapheim KM, Pan H, Li C, Salzberg SL, Puiu D, Magoc T, Robertson HM, Hudson ME, Venkat A & Fischman BJ (2015) Genomic signatures of evolutionary transitions from solitary to group living. Science 348: 1139-1143.

Kearns CA, Inouye DW & Waser NM (1998) Endangered mutualisms: the conservation of plant-pollinator interactions. Annual Review of Ecology and Systematics: 83-112.

Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C & Tscharntke T (2007) Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences 274: 303-313.

Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R & Popov S (1999) Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. Journal of Ethnopharmacology 64: 235-240.

Langstroth LL (1914) Langstroth on the Hive and the Honey-bee: A Bee-keeper's Manual. Hopkins, Bridgman & Company.

Lee KV, Steinhauer N, Rennich K, Wilson ME, Tarpy DR, Caron DM, Rose R, Delaplane KS, Baylis K & Lengerich EJ (2015) A national survey of managed honey bee 2013–2014 annual colony losses in the USA. Apidologie 46: 292-305.

Losey JE & Vaughan M (2006) The economic value of ecological services provided by insects. Bioscience 56: 311-323.

Martin SJ, Highfield AC, Brettell L, Villalobos EM, Budge GE, Powell M, Nikaido S & Schroeder DC (2012) Global honey bee viral landscape altered by a parasitic mite. Science 336: 1304-1306.

Masterman R, Ross R, Mesce K & Spivak M (2001) Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L.). Journal of Comparative Physiology A 187: 441-452.

Masterman R, Smith B & Spivak M (2000) Brood odor discrimination abilities in hygienic honey bees (*Apis mellifera* L.) using proboscis extension reflex conditioning. Journal of Insect Behavior 13: 87-101.

Meixner MD (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology 103: S80-S95.

Memmott J, Waser NM & Price MV (2004) Tolerance of pollination networks to species extinctions. Proceedings of the Royal Society of London. Series B: Biological Sciences 271: 2605-2611.

Menzel R (2012) The honeybee as a model for understanding the basis of cognition. Nature Reviews Neuroscience 13: 758-768.

Möckel N, Gisder S & Genersch E (2011) Horizontal transmission of deformed wing virus: pathological consequences in adult bees (*Apis mellifera*) depend on the transmission route. Journal of General Virology 92: 370-377.

Morse RA & Calderone NW (2000) The value of honey bees as pollinators of US crops in 2000. Bee Culture 128: 1-15.

Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R & Pettis JS (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS ONE 5: e9754.

Naug D (2009) Nutritional stress due to habitat loss may explain recent honeybee colony collapses. Biological Conservation 142: 2369-2372.

Nazzi F, Brown SP, Annoscia D, Del Piccolo F, Di Prisco G, Varricchio P, Della Vedova G, Cattonaro F, Caprio E & Pennacchio F (2012) Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathogens 8: e1002735.

Neumann P & Carreck NL (2010) Honey bee colony losses. Journal of Apicultural Research 49: 1-6.

Page RE, Robinson GE, Fondrk MK & Nasr ME (1995) Effects of worker genotypic diversity on honey bee colony development and behavior (*Apis mellifera* L.). Behavioral Ecology and Sociobiology 36: 387-396.

Peng Y-SC, Fang Y, Xu S, Ge L & Nasr ME (1987) Response of foster Asian honeybee (*Apis cerana Fabr*.) colonies to the brood of European honeybee (*Apis mellifera L.*) infested with parasitic mite, *Varroa jacobsoni* Oudemans. Journal of Invertebrate Pathology 49: 259-264.

Pettis JS, Collins AM, Wilbanks R & Feldlaufer MF (2004) Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. Apidologie 35: 605-610.

Pettis JS & Delaplane KS (2010) Coordinated responses to honey bee decline in the USA. Apidologie 41: 256-263.

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O & Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. Trends in Ecology & Evolution 25: 345-353.

Rath W & Drescher W (1990) Response of *Apis cerana* Fabr. towards brood infested with *Varroa jacobsoni* Oud. and infestation rate of colonies in Thailand. Apidologie 21: 311-321.

Rosenkranz P, Aumeier P & Ziegelmann B (2010) Biology and control of *Varroa destructor*. Journal of Invertebrate Pathology 103: S96-S119.

Rosenkranz P, Tewarson NC, Singh A & Engels W (1993) Differential hygienic behaviour towards *Varroa jacobsoni* in capped worker brood of *Apis cerana* depends on alien scent adhering to the mites. Journal of Apicultural Research 32: 89-93.

Rueppell O, Hayworth M & Ross N (2010) Altruistic self‐removal of health‐compromised honey bee workers from their hive. Journal of Evolutionary Biology 23: 1538-1546.

Rueppell O, Pankiw T, Nielsen DI, Fondrk MK, Beye M & Page RE (2004) The genetic architecture of the behavioral ontogeny of foraging in honeybee workers. Genetics 167: 1767-1779.

Schatton-Gadelmayer K & Engel W (1988) Blood proteins and body weight of newly-emerged worker honeybees with different levels of parasitization of brood mites (Hymenoptera: Apidae: *Apis mellifera*/Acarina: Varroidae: *Varroa jacobsoni*). Entomologia Generalis 14: 93-101.

Shi YY, Huang ZY, Zeng ZJ, Wang ZL, Wu XB & Yan WY (2011) Diet and cell size both affect queen-worker differentiation through DNA methylation in honey bees (*Apis mellifera*, Apidae). PLoS ONE 6: e18808.

Simone M, Evans JD & Spivak M (2009) Resin collection and social immunity in honey bees. Evolution 63: 3016-3022.

Spivak M (1996) Honey bee hygienic behavior and defense against *Varroa jacobsoni*. Apidologie 27: 245-260.

Spivak M, Mader E, Vaughan M & Euliss Jr NH (2011) The Plight of the Bees. Environmental Science & Technology 45: 34-38.

Spivak M, Masterman R, Ross R & Mesce KA (2003) Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. Journal of Neurobiology 55: 341-354.

Spivak M & Reuter GS (2001) Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. Apidologie 32: 555-565.

Spleen AM, Lengerich EJ, Rennich K, Caron D, Rose R, Pettis JS, Henson M, Wilkes JT, Wilson M & Stitzinger J (2013) A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. Journal of Apicultural Research 52: 44-53.

Starks PT, Blackie CA & Seeley TD (2000) Fever in honeybee colonies. Naturwissenschaften 87: 229-231.

Stefanidou M, Athanaselis S & Koutselinis A (2003) The toxicology of honey bee poisoning. Veterinary and Human Toxicology 45: 261-265.

Steinhauer N, Rennich K, Lee KV, Pettis J, Tarpy DR, Rangel J, Caron D, Sagili R, Skinner JA, Wilson ME, Wilkes JT, Delaplane KS, Rose R & van Engelsdorp D (2015) Colony Loss 2014-2015: Preliminary Results, Vol. 2015.

Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS, Rose R, Skinner JA, Tarpy DR & Wilkes JT (2014) A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. Journal of Apicultural Research 53: 1-18.

Sylvester HA, Watts RP, De Guzman L, Stelzer JA & Rinderer TE (1999) *Varroa* in the mating yard: II. The effects of *Varroa* and fluvalinate on drone mating competitiveness. American Bee Journal.

vanEngelsdorp D, Caron D, Hayes J, Underwood R, Henson M, Rennich K, Spleen A, Andree M, Snyder R & Lee K (2012) A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. Journal of Apicultural Research 51: 115-124.

vanEnglesdorp D, Hayes Jr J, Underwood RM & Pettis J (2008) A survey of honey bee colony losses in the US, Fall 2007 to Spring 2008. PLoS ONE 3: e4071.

Waddington KD & Rothenbuhler WC (1976) Behaviour associated with hairless-black syndrome of adult honeybees. Journal of Apicultural Research 15: 35-41.

Weinstock GM, Robinson GE, Gibbs RA, Worley KC, Evans JD, Maleszka R, Robertson HM, Weaver DB, Beye M & Bork P (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443: 931-949.

Wenner A & Bushing W (1996) *Varroa* mite spread in the United States. Bee Culture 124: 341-343.

Winston ML (1991) The Biology of the Honey Bee. Harvard University Press, Cambridge, Massachusetts.

Yang X & Cox-Foster DL (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proceedings of the National Academy of Sciences of the United States of America 102: 7470-7475.

CHAPTER II

EFFECTS OF BROOD TYPE ON HYGIENIC REMOVAL IN THE HONEY BEE *(APIS MELLIFERA)*

This chapter is coauthored by Kaira M. Wagoner and Olav Rueppell.

Introduction

Communication is essential for facilitation of cooperation between conspecific members of social species. In social insects, chemical signals are often used to communicate information such as family membership, fecundity, danger, territoriality, and resource quality and location. Within social insect family groups, chemical signals from the young, developing generation (brood) are important for regulation of nest maintenance and resource allocation. Though theories regarding the effects of brood chemicals on adult behavior have been around for over a century (Wheeler, 1910), worker attraction to brood chemicals was first demonstrated in the army ant *Neivamyrmex opacithorax*, in 1966 (Watkins & Cole). Since then, studies of numerous social insect species have demonstrated or indicated the potential for relationships between brood chemicals - especially hydrocarbons, phenols and fatty aliphatic esters - and a variety of adult behaviors. For example, in termites, brood chemicals have been implicated in corpse burial (Chouvenc et al., 2012) and nest building (Ulyshen & Shelton, 2012). The close relationship between brood chemicals and adult behavior has also been demonstrated in many hymenopterans. In ants, chemicals have been implicated in the

recognition of brood caste (Brian, 1975), corpse management (Choe et al., 2009; Sun & Zhou, 2013), brood cannibalism (Monnin & Peeters, 1997), and brood relocation (Glancey et al., 1970; Walsh & Tschinkel, 1974). In honey bees, chemicals have been implicated in acceptance and feeding of queen cells (Le Conte et al., 1995), cell capping (Le Conte et al., 1990; Swanson et al., 2009), ratio of nectar to pollen foraged (Traynor et al., 2015), diploid drone cannibalism (Santomauro et al., 2004). In honey bees, brood chemicals have also been implicated (Free & Winder, 1983) or insinuated (Aumeier & Rosenkranz, 2001; Swanson et al., 2009) in the induction of hygienic behavior.

Hygienic behavior is defined as the detection and removal of diseased brood from a colony or nest (Spivak & Reuter, 2001a; Wilson-Rich et al., 2009). While hygienic behavior is thought to occur in the dampwood termite *Zootermopsis angusticollis* and the leaf-cutting ant *Atta sexdens rubropilosa* (Wilson-Rich et al., 2009), it has been studied primarily in the honey bee *Apis mellifera*. Like other eusocial insects, honey bees are especially susceptible to disease due to high contact rates with genetically similar nestmates (Cremer et al., 2007). The honey bee serves as a good model for the study of hygienic behavior because of its prevalence, its domestication, and the existence of hygienic behavior breeding programs that have increased the trait's frequency within certain honey bee lines. Honey bees are also important to study because, despite the presence of natural honey bee social immune mechanisms like hygienic behavior, honey bee health is currently being severely threatened. Following global pollinator population trends (Genersch et al., 2010; Meixner, 2010; Potts et al., 2010), managed honey bee colonies in the United States have declined steadily for over six decades, from 5.9 million

colonies in 1947 to 2.4 million colonies in 2005 (Meixner, 2010; Pettis & Delaplane, 2010). Total annual colony losses in the United States have exceeded 33% in four of the last five years, exceeding 45% between April 2012 and April 2013 (Lee et al., 2015; Spleen et al., 2013; Steinhauer et al., 2015; Steinhauer et al., 2014; vanEngelsdorp et al., 2012).

Recent honey bee losses are largely attributed to the introduction and spread of new parasites and associated pathogens, and to lethal and sublethal effects of agrochemical exposure (Lee et al., 2015; Nazzi et al., 2012; Potts et al., 2010). *Varroa destructor* (*Varroa*) is an obligate, ectoparasitic honey bee mite, arguably the most important threat to honey bee health and apiculture today (Anderson & Trueman, 2000; Rosenkranz et al., 2010). Originally a parasite of the Asian honey bee *Apis cerana, Varroa* made a host-shift to the European honey bee *Apis mellifera* in Asia about four decades ago (Anderson & Trueman, 2000) and was first identified in the United States in 1987 (de Guzman & Rinderer, 1999). During their reproductive stage, female foundress mites enter honey bee brood cells just before capping and bury themselves in the brood food at the base of the cell. After about six hours, when the food has been consumed by the bee brood, the mite emerges and establishes a feeding site on the brood, from which it sucks hemolymph (Ifantidis, 1988). Approximately 70 hours after cell capping the foundress mite begins to lay eggs, the first of which is haploid and develops into a male. Diploid eggs are then laid at approximately 30-hour intervals. These develop into females which mate with the waiting male such that by the end of honey bee development up to four (worker cell) or five (drone cell) fertilized female *Varroa* may emerge with the

emerging honey bee to repeat the cycle (Rosenkranz et al., 2010). *Varroa* act as a physical burden to the bee, reducing body weight and protein levels primarily through the sucking of hemolymph (Amdam et al., 2004; D'Aubeterre et al., 1999; De Jong et al., 1982; Garedew et al., 2004; Schatton-Gadelmayer & Engel, 1988). While the physical burden of *Varroa* is problematic to honey bee health, it is merely one of many honey bee threats associated with *Varroa*. *Varroa* transmit pathogens to honey bees (Bowen-Walker et al., 1999; Chen et al., 2004; Kanbar & Engels, 2003; Martin et al., 2012) and have been associated with both viral amplification and honey bee disease susceptibility (Martin et al., 2012; Yang & Cox-Foster, 2005). Additionally miticides such as fluvalinate and coumaphos, applied directly to colonies to control *Varroa* infestations, have harmful effects on honey bee queens, workers, and drones. For example, moderate doses of fluvalinate in the hive have been associated with reduced queen weight (Haarmann et al., 2002), worker mortality (Pettis et al., 1991), and reduced drone weight and number of spermatozoa (Sylvester et al., 1999). Even low doses of coumaphos have been associated with increased queen mortality, physical deformities, reduced body and ovary weight, and atypical behavior (Collins et al., 2004; Haarmann et al., 2002; Pettis et al., 2004) and moderate coumaphos exposure has been linked to reduction of drone sperm vitality (Burley et al., 2008). The lipophilic nature of both the synthetic pyrethroid fluvalinate and the organophosphate coumaphos leads to high absorption and accumulation of the chemicals in hives, especially in wax, meaning that exposure of bees to these and similar compounds increases with time and number of chemical treatments. A 2007 study of residues in honey bee colonies found 46 pesticides in 108 pollen samples, and 20

pesticides in 88 wax samples, with over 55% of pollen and 100% of wax samples containing the most concentrated pesticides: the miticides fluvalinate and coumaphos (Frazier et al., 2008).

Despite substantial evidence of the need, no adequate solution for control of *Varroa* has been developed (Dietemann et al., 2012). Miticides used for protection against *Varroa* are harmful to bees and lose effectiveness as *Varroa* become resistant (Rosenkranz et al., 2010; Sammataro et al., 2005), leading in some cases to use of unauthorized chemicals by beekeepers desperate to control the pest (Johnson et al., 2010). According to an analysis of honey bee chemical susceptibility and the physiological targets of existing miticides, development of miticides with novel active ingredients is unlikely (Dekeyser & Downer, 1994; Rosenkranz et al., 2010). While other control strategies such as physical mite removal and use of essential oils do exist, they have many limitations that compromise efficacy, including increased labor for the beekeeper (and thus lack of uptake), temperature vulnerability, and minimal differences between target and non-target lethal dose concentrations (Dietemann et al., 2012; Imdorf et al., 2003).

One especially promising solution to the problem of *Varroa* is the augmentation of colony resistance through the selective breeding of hygienic behavior (Boecking et al., 2000; Dietemann et al., 2012; Rinderer et al., 2010; Spötter et al., 2012; Tsuruda et al., 2012). Due to the economic importance of hygienic traits, *Varroa* resistance through hygienic behavior has been successfully selected for in honey bees. The Minnesota Hygienic (HYG) line was selected for using freeze-killed brood (FKB) assays, which

quantify a colony's level of hygiene by the time required for removal of approximately 200 freeze-killed brood (Spivak, 1996). The *Varroa* Sensitive Hygienic (VSH) line was originally selected for using a distinct phenotype, the suppression of mite reproduction (SMR) (Harbo & Harris, 2001). Although it is unclear whether mite resistance in the VSH line is a result of the interruption of the mite reproductive cycle by hygienic adults or of some unknown mechanism of the brood, invading *Varroa* mites have limited reproductive success in VSH colonies compared to HYG colonies (Harbo & Harris, 2005; Ibrahim & Spivak, 2006). Both HYG and VSH breeds have been shown to be more resistant to *Varroa* mites compared to unselected control bees, (Danka et al., 2013; Harris, 2007; Harris et al., 2012; Spivak & Gilliam, 1998; Spivak & Reuter, 2001a; b) though VSH bees remove a greater amount of mite-infested brood than do HYG bees (Danka et al., 2013; Ibrahim & Spivak, 2006).

Despite the relative success of these breeding strategies, hygienic lines do not currently serve as sustainable alternatives to chemical *Varroa* control (Dietemann et al., 2012), as chemical treatments are still required to control severe mite infestations in hygienic colonies (Ibrahim et al., 2007; Spivak & Reuter, 2001b). Though horizontal transmission of *Varroa* from non-hygienic colonies may be partially to blame for infestations in hygienic colonies, other reasons for lack of sustainability include non mite-specificity of selection processes (Pernal et al., 2012; Rinderer et al., 2010; Spotter et al., 2012), expense of current selection assays (Espinosa-Montano, 2008), and lack of uptake by the beekeeping community (Carreck, 2011; Espinosa-Montano, 2008). The first of these issues is of crucial importance as the olfactory trigger for hygienic removal

of mite-infested brood is apparently lower than that of dead or highly infected brood (Boecking & Drescher, 1992; Spivak & Downey, 1998).

While the majority of studies of hygienic behavior mechanisms have focused on sensitivity and modulation of adult honey bee olfaction (Aumeier & Rosenkranz, 2001; Goode et al., 2006; Harbo & Harris, 2009; Ibrahim & Spivak, 2006; Masterman et al., 2001; Masterman et al., 2000; Spivak et al., 2003) recent evidence indicates the importance of olfactory signals in influencing honey bee hygienic behavior in relation to detection and removal of mite infested brood, specifically. Though a limited number of studies have provided evidence that olfactory signals originate from the mite (Martin et al., 2002), or at least around the time of mite oviposition (Harbo & Harris, 2009), other studies have rejected the notion that the mite itself is the trigger for removal (Aumeier & Rosenkranz, 2001; Harris et al., 2010). Furthermore, several studies suggest that mites are able to mimic host odor profiles, likely through passive camouflage (Kather et al., 2015), with such accuracy that even developmental-stage (Martin et al., 2001) and colony-specific differences (Le Conte et al., 2015) are accounted for. This and evidence of differences in chemical brood profiles associated with varying degrees of brood health and parasitism point towards chemical signals originating from the honey bee brood themselves. For example, Swanson et al. (2009) identified three volatile compounds associated with chalkbrood-infected larvae, which were absent from larvae not infected with the fungus. Another recent study linked removal behavior with mite virulence, where brood parasitized by highly virulent mites were more likely to be removed than those parasitized by less virulent mites (Schöning et al., 2012). This study also found

differences in the chemical profiles of brood infested with mites of high and low Deformed Wing Virus (DWV) virulence, suggesting that olfactory cues may drive damage-dependent removal of honey bee brood (Schöning et al., 2012). The presence of mites, regardless of their virulence, has also been associated with changes in brood chemical profiles (Salvy et al., 2001). Based on this collection of evidence we hypothesized that, in addition to its well-established influence on adult olfaction, selection for hygienic behavior in honey bees has influenced brood signaling. More specifically, we tested the predictions that honey bee brood removal rates are affected by 1) brood type and 2) hygiene level of the brood's colony of origin. To test this hypothesis we performed in-hive behavioral assays to determine the effect of interactions between various adult and brood types of varying *Varroa* mite resistance on hygienic removal behavior. Results from this study reveal the importance of brood type and the hygiene level of the brood's colony of origin in influencing hygienic removal behavior in the honey bee, and suggest that brood signaling may be an important mechanism by which various adult behaviors are triggered in honey bees and other eusocial species.

Materials and Methods

Overview

Over two consecutive summers, sections of honey bee combs containing eggs from queens of different sources whose workers displayed various levels of hygienic behavior were combined together within one frame. These frames, containing eggs from multiple queens, were placed into unrelated colonies for rearing, such that no egg went

back into its colony of origin. *Varroa* mite, wound and control treatments were applied. Removal status of each brood cell over a one-week period was recorded. This experimental setup allowed comparison of removal of different treatment groups 1) between brood types within each colony type, and 2) for each brood type across colony types.

Materials

Wooden frames, wax foundation, and unselected Italian control (CON) queens were purchased from Triad Bee Supply in Trinity, NC. Minnesota Hygienic (HYG) queens were donated by Jeff Hull and Amy Weeks in West Monroe, LA. *Varroa* Sensitive Hygienic (VSH) queens were donated by Bob Danka at the United States Department of Agriculture's Agricultural Research Center (USDA-ARC) in Baton Rouge, LA. All queens were open mated and studied for one bee season only. Sample sizes for colonies of CON, HYG and VSH origin were 2, 4 and 3, respectively for 2013, and 2, 2 and 2, respectively for 2014. Colonies were established by introducing marked queens to queenless colonies, and waiting a minimum of 7 weeks from the time of introduction before initiating experiments. A 7 week waiting period was selected because worker development time from egg to adult emergence requires approximately 21 days (Bertholf, 1925), and hygienic behavior is most commonly observed in workers aged 15 to 18 days post-emergence (Arathi et al., 2000). This wait period also allows for the death of most preexisting workers, which have lifespans in the summer of between approximately 25 and 35 days (Free & Spencer‐Booth, 1959; Maurizio & Hodges, 1950). Directly

following the behavioral experiments, freeze-killed brood (FKB) assays were performed to determine the level of hygienic behavior exhibited by each colony (Spivak & Downey, 1998). However, due to insufficient brood frames, successful FKB assay results were only obtained for 11 of the 15 colonies tested.

Methods

All behavioral assays were conducted at the University of North Carolina at Greensboro bee-yard during the summers of 2013 and 2014. Each year, medium wooden frames were sawed vertically into equal thirds and reassembled using metal brackets or staples. Reassembled frames were fitted with new wax foundation containing steel vertical wire stabilizers. Frames were placed into the top box of unselected CON colonies, above queen excluders, so that the comb could be drawn out but the queen could not lay eggs in the cells.

Once combs were drawn out, frames were removed from unselected colonies and fitted with wire cages constructed using mesh size 0.5cm x 0.5cm, such that workers could move freely on and off both sides of each frame but the queen, once introduced, could not leave. A single queen of unselected control (CON), Minnesota Hygienic (HYG), or *Varroa*-Sensitive Hygienic (VSH) origin was placed on each frame. Caged experimental frames were returned to each queen's respective colony. Once eggs were present on >75% of both sides of a frame, frames were removed from their colonies. A razor blade was used to cut combs into thirds (corresponding to the previous frame cuts) and metal brackets and staples were removed. Sections from different frames were then

grafted together using metal brackets and staples such that each new frame contained eggs from at least two queens of varying (VSH, HYG or CON) origin. Frames were then redistributed into new colonies, such that no brood was ever placed back into its colony of origin.

After allowing 5-7 days for development, the location of uncapped cells containing 5th larval instars were marked using a permanent marker and transparent plastic sheet secured over each frame with thumb tacks. Cells along wires were avoided since wires are associated with increased brood removal rates (see Chapter IV). Frames were returned to their colonies for 12 to 16 hours and then checked to ensure that they had been sealed with a wax cap for the initiation of pupation. This procedure ensured that treatments were administered to capped cells within 18 hours of capping, as is necessary to ensure initiation of mite oogenesis (Frey et al., 2013). Mite, wound and control treatments were administered to recently capped cells (Kuster et al., 2014) in each section of frame corresponding to a different breed. To open the caps of experimental cells, one side of the cap was cut with the edge of a razor blade. The cell cap could then be lifted up, and resealed after treatment administration by pressing the cell cap against the cell wall with the edge of the razor. The treatments assigned to each cell were selected at random, and each cell received either one mite, one wound, or the control treatment. Mites were collected from non-experimental colonies at the UNCG bee-yard using the sugar shake method (Dietemann et al., 2013; Fakhimzadeh, 2001). Mites were shaken on to a damp paper towel, and were gently rinsed with a drop of clean water before being introduced to cells using a fine-tipped paintbrush within approximately one hour of collection. Mites

that could not clutch the paintbrush bristles were considered to be unhealthy, and were not used. Wounds that mimic *Varroa* mite feeding were inflicted within the brood cell using 50μm diameter capillary needles on the dorsal side of the brood between the first abdominal segment and the second thoracic segment according to existing protocols (Dade, 2009; Herrmann et al., 2005). Note that this wound is non-lethal, distinct from the pin-killed brood bioassay used for selection of hygienic behavior (Newton & Ostasiewski, 1986). Control cells were opened and resealed just as mite and wound cells, but received neither mite nor wound treatment. In 2013, a total of 1,063 cells were included in the study (349 VSH, 462 HYG, and 252 CON cells from 2, 4 and 3 colonies, respectively), and in 2014, an additional 1,025 cells were included (320 VSH, 354 HYG, 351 CON cells, from 2, 2, and 2 colonies, respectively).

Each day for one week following treatment administration, experimental frames were removed from their colonies for no more than 30 minutes per day to allow monitoring of each experimental cell for uncapping and removal. Brood removed on the first day following treatment introductions was excluded to avoid experimental artifacts, such as removal triggered by poorly resealed cells.

Statistical Analysis

Overall effects of treatment, colony type and brood type on removal rates were assessed independently from each other using separate Chi-square analyses for each year. To facilitate comparison with past studies that did not cross-foster brood, Chi-square tests were used to assess the effect of colony type on brood removal rate for the subset of data

in which colony type was the same as brood type. To better understand the relationship between brood type and removal of the *Varroa* mite, Chi-square tests were also used to assess the brood effects for removal of mite-infested cells alone. A full factorial logistic regression model was then used to determine the individual and interactive effects of treatment, colony type, brood type, and year on brood removal. This analysis revealed higher order interactions (see results) that made the full data set difficult to interpret. Chi-square tests were used to determine the effect of brood type on removal (the focus of our hypothesis) for each treatment by colony type by year combination. Bonferroni correction was used for pairwise comparisons to control the family-wise error rate within each treatment by colony type by year combination (using a corrected significance threshold of $p \leq 0.0167$).

For the 11 colonies with FKB assay data, Pearson product-moment correlation coefficients were computed to assess the relationships between 1) average percentage removal of brood in relation to the level of hygiene of the brood's colony of origin (overall and for each treatment type: mite, wound, or control), and 2) average percentage removal of brood in relation to the level of hygiene of its host colony (overall and for each treatment type: mite, wound, or control). All statistical analyses were performed using IBM SPSS Statistics, Version 22.0.0.0.

Results

Effects of Treatment, Colony Type and Brood Type on Removal Rates

The initial evaluation of overall effects of treatment, colony type, and brood type revealed significant effects of each factor across the different experimental levels of the other factors. Treatment had a significant effect on brood removal rate in 2013 and 2014 (2013: χ^2=31.3, d.f.=2, p<0.001; 2014: χ^2=73.4, d.f.=2, p<0.001; Figure 1). Colony type had a significant effect on brood removal rate in 2013 but not in 2014 (2013: χ^2=61.1, d.f.=2, p<0.001; 2014: χ^2=2.7, d.f.=2, p=0.263; Figure **2**). In the subset of data for which colony type was the same as brood type, colony type had a significant effect on brood removal rate in 2013 and 2014 (2013: χ^2=28.3, d.f.=2, p<0.001; 2014: χ^2=10.5, d.f.=2, p=0.005; Figure 3). Brood type had a significant effect on brood removal rate in 2013 and 2014 in both the full data set (2013: χ^2=31.4, d.f.=2, p<0.001; 2014: χ^2=42.1, d.f.=2, p<0.001; Figure 4) and for mite-infested cells only (2013: χ^2=14.6, d.f.=2, p=0.001; 2014: χ^2=30.7, d.f.=2, p<0.001; Figure 5). The full logistic regression model, including the effects of brood type, colony type, treatment, year and all interactions on brood removal, was statistically significant (χ^2=328.1, df=53, p<0.001; Figure 6). The model achieved a correct classification of brood removal in 83.3% of the cases with an associated Nagelkerke's R^2 of 0.24. Multiple factors and interactions were statistically significant (Table 1). In order to understand our results in detail with respect to our main hypothesis, the effects of brood type on removal were evaluated for each treatment by colony type by year combination. These analyses revealed the importance of brood type

on removal in most contexts when the brood was injured or mite parasitized, although effects varied between years (Figure **6**).

Effects of Hygiene Level on Removal Rate

Freeze-killed brood (FKB) assays were used to determine hygiene level for individual colonies (Table 2). A significant, positive correlation was identified between removal of brood (regardless of treatment or the host colony type) and the level of hygiene displayed by bees from the brood's colony of origin (r=.680, n=11, p=0.021). That is, brood that originated from highly hygienic colonies (as determined by FKB assays) was more likely to be removed than brood that originated from non-hygienic colonies when all treatments and host colony types were considered. No comparable correlation was identified between removal of brood (regardless of treatment or the brood's colony of origin) and the level of hygiene of the brood's host colony (r=-.414, n=11, p=0.205). In other words, brood hosted in highly hygienic colonies (as determined by FKB assays) was not more or less likely to be removed than brood hosted in non-hygienic colonies when all treatments and brood types were considered.

The relationships between hygiene level and removal rate were further explored through separate correlation analyses for each treatment. For the mite treatment, there was a significant positive correlation between percent brood removal and the percent of freeze-killed brood removed by bees in the brood's colony of origin (r=0.658, n=11, p=0.028; Figure **7**A). However no significant correlation was found for corresponding wound (r=0.563, n=11, p=0.072) or control (r=0.455, n=11, p=0.160) treatments (

Figure **7**A). In other words, for brood originating from highly hygienic colonies, only those receiving the mite treatment were more likely to be removed when removal by all host colony types was considered. For the wound treatment, there was a significant negative correlation between percent brood removal and the percent of freeze-killed brood removed by bees in the brood's host colony ($r=-0.766$, $n=11$, $p=0.006$; Figure **7**B). No significant correlation was found for corresponding mite ($r=-0.134$, $n=11$, $p=0.694$) or control ($r=-0.389$, $n=11$, $p=0.237$) treatments (Figure **7**B). In other words, for brood hosted by highly hygienic colonies, only those receiving the wound treatment were less likely to be removed when removal of all brood types was considered.

Discussion

In an effort to determine whether selection for hygienic behavior in the honey bee has influenced brood signaling, we cross-fostered mite-infested and control brood among three honey bee colony types to determine the effect of interactions between various adult and brood types with respect to hygienic removal behavior. Our hypothesis that hygienic removal is colony-type specific was only supported by 2014 data; VSH brood was more likely to be removed than HYG brood in 2014, despite similarities in VSH and HYG hygiene levels for that year. Our hypothesis that hygienic behavior is influenced by brood signals was supported by data from both years; overall, brood from colonies considered highly hygienic, based on the freeze-killed brood assay, was removed more readily by all colony types (CON, HYG and VSH) than brood from less hygienic colonies.

The overall effect of treatment on brood removal in our study (Figure 1) was similar to findings from previous studies (Harris et al., 2010; Spivak, 1996), both in terms of absolute and relative removal rates of mite-infested and control brood. Differences in the rates of mite-infested brood removal by their own colony types (Figure **6**) were also consistent with previous research, indicating that VSH colonies tend to remove more mite-infested brood of their own breed than do HYG colonies (Danka et al., 2013; Ibrahim & Spivak, 2006), and that, with the exception of HYG colonies in 2013, colonies selected for hygienic behavior remove more mite-infested brood than do unselected colonies (Danka et al., 2013; Harbo & Harris, 2005; Spivak & Reuter, 2001b; Toufailia et al., 2014). The unexpectedly low removal rates of all brood types exposed to all treatment types by two of the 2013 HYG colonies, despite their testing as 99 and 100% hygienic in the FKB assays, suggests that these colonies may have been health-compromised in a way that was not visually apparent at the time; e.g., they may have been infected with one or more honey bee viruses (Chen & Siede, 2007). This possibility is supported by the elevated removal rates of HYG brood in 2013 (Figure 4). Another possible and potentially concomitant explanation for low brood removal by HYG colonies in 2013 is the inconsistency between FKB removal and mite removal (Spivak, 1996; Spivak & Gilliam, 1998). While removal of FKB is related to removal of *Varroa* (Toufailia et al., 2014), FKB present a stronger removal stimulus than mite-infested brood (Boecking & Drescher, 1992; Spivak & Downey, 1998). As a result, FKB removal tends to be less variable than removal of mite-infested brood (Danka et al., 2013; Spivak, 1996), and it is

possible that bees in the 2013 HYG colonies had high thresholds for abnormal brood odors relative to other colonies.

The significant effects of brood type on brood removal rate for 2014, illustrated in Figure 4 and Figure 5, support our hypothesis that hygienic removal is colony-type specific, as VSH brood was more likely to be removed than HYG brood in 2014 despite similarities in VSH and HYG hygiene levels for that year (Table 2). Though HYG brood was more likely to be removed than VSH and CON brood in 2013, this may have been an effect of hygiene level (according to the FKB assay) rather than a colony-type specific effect, as average VSH hygiene level was unexpectedly low in 2013 (Table 2). Ç

Our hypothesis that adult hygienic behavior is influenced by brood signals was supported by the significant positive correlation observed between the percent brood removal and the percent of freeze-killed brood removed by bees in the brood's colony of origin (Figure **7**A). Though the positive correlations illustrated in Figure **7**A were only significant for mite-infested cells, the overall trends suggest that the more hygienic the brood's colony of origin, the more likely that brood was to be removed. In contrast, though the negative correlations illustrated in Figure **7**B were only significant for wounded brood, the overall trends suggest that the more hygienic the brood's host colony, the less likely that brood was to be removed. In a real colony setting, this effect would likely be negated, because brood and adults of the same breed would be in the same colony. However this may suggest that the more hygienic an adult is, the more likely she is to be a specialist in terms of what signals influence her to initiate removal behavior. Furthermore there may be some sort of co-evolution between signal intensity and receiver

sensitivity, where the more pronounced one ability is, the weaker the other. This is consistent with response threshold models, which predict that increasing stimulus strength leads to detection from individuals with higher thresholds, and thus leads to greater genotypic variety among individuals involved in stimulus response (Beshers & Fewell, 2001; Fewell & Page Jr, 1993). Consequently, hygienic colonies capable of producing strong brood signals in response to mite-infestation would need relatively fewer adults with enhanced olfaction capabilities, and mite detection would tend to be carried out by the genetic subset of individuals with the lowest thresholds, while the removal of mite-infested brood would be carried out by individuals with higher thresholds (Arathi et al., 2000; Gramacho & Spivak, 2003; Spivak et al., 2003).

To our knowledge, this is the first behavioral study of honey bee hygiene to cross-foster various brood types. In previous behavioral studies of honey bee hygiene in which brood are not cross-fostered, it is difficult to distinguish whether measured hygienic behaviors are a result of differences in brood signaling or enhanced adult olfaction (Harbo & Harris, 2005; Ibrahim & Spivak, 2006; Spivak, 1996; Spivak & Reuter, 2001b). Our study's lack of evidence for a significant positive correlation between the percent brood removal and the percent of freeze-killed brood removed by adult bees in the brood's host colony (Figure **7**B) is likely an artifact of cross-fostering, as analysis of colony type effects where brood type was the same as colony type (Figure **3**) correspond to previous findings suggesting that hygienic behavior relies on the sensitivity and modulation of adult honey bee olfaction (Martin et al., 2002; Masterman et al., 2001; Spivak et al., 2003). In other words, cross-fostering allowed us to differentiate the role of

brood signaling from the role of adult signal perception. Our study's evidence for brood signaling is also supported by findings from several previous studies, including studies presenting evidence that, although hygienic behavior takes place around the time of mite oviposition (Harbo & Harris, 2009), hygienic behavior is not affected by *Varroa* movement (Aumeier & Rosenkranz, 2001), scent (Aumeier & Rosenkranz, 2001; Le Conte et al., 2015), or offspring (Harris et al., 2010). Previous studies have also provided evidence that cuticular hydrocarbons are altered in mite-infested honey bee brood (Annoscia et al., 2012; Salvy et al., 2001; Schöning et al., 2012). Additionally, a study published in 2015 found that hydrolase activity, specifically that related to cytochrome P450 pathways, was over-expressed in the brains of non-hygienic bees (Boutin et al.). Since P450s are involved in the breakdown of chemicals including pheromones (Feyereisen, 1999), the authors of the study proposed that overexpression of cytochrome P450s in non-hygienic honey bees may lead to the breakdown of chemical triggers for hygienic behavior (Boutin et al., 2015), further supporting the theory that hygienic breeds are more effective than unselected breeds at brood signaling. Several other studies also indicate genetic and/or proteomic differences between brood from hygienic and non-hygienic colonies (Boutin et al., 2015; Le Conte et al., 2011; Navajas et al., 2008; Parker et al., 2012; Tsuruda et al., 2012). It is important to note that the theories of enhanced brood signaling and enhanced adult detection are not mutually exclusive. However if brood signals are more important to hygienic behavior than adult olfaction as our results suggest, our findings may explain why the theory of increased olfactory sensitivity in

hygienic honey bees is not supported by the roles of candidate genes previously associated with hygienic behavior (Le Conte et al., 2011).

Threats to honey bee health are of great importance, especially considering the value of this pollinator's contributions to food security (Aizen et al., 2008; Aizen & Harder, 2009; Holden, 2006; Kearns et al., 1998; Klein et al., 2007) and maintenance of natural ecosystems (Memmott et al., 2004). Novel control methods for *Varroa* and other honey bee diseases are urgently needed, and one sustainable and environmentally responsible option is to further develop biological strategies such as selective breeding of pest- and disease-resistant honey bees. *Varroa*-resistant bees do not require the time and expense of new compound development and approval, and do not lead to development of resistance or pesticide exposure and accumulation in bees, humans or ecosystems (Dietemann et al., 2012; Rinderer et al., 2010). The evidence for colony-type specific brood signals presented here represents a critical step towards the improvement of breeding strategies for *Varroa*-resistant honey bees. For example, improved signaling capabilities and lower thresholds of disease and/or *Varroa* detection may be achieved by basing honey bee selection on an olfactory trigger released by hygienic brood, rather than using high threshold indicators such as FKB. Our results predict existing distinctions between HYG and VSH brood signaling abilities, and encourage further studies designed to identify the chemical nature of colony-type specific honey bee brood signals (see Chapter III).

The improvement of honey bee health resulting from improved *Varroa*-resistance and decreased exposure to harmful chemicals commonly use to treat for *Varroa*

infestations will lead to increased yield of honey bee products, increased food security through improvement in crop yields, and increased health of natural ecosystems. Furthermore, improved understanding of nestmate interactions may facilitate development of novel strategies to combat other honey bee threats, and could lead to important insights regarding communication between members of other social insects, including both beneficial and pest species.

References

Aizen MA, Garibaldi LA, Cunningham SA & Klein AM (2008) Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. Current Biology 18: 1572-1575.

Aizen MA & Harder LD (2009) The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Current Biology 19: 915-918.

Amdam GV, Hartfelder K, Norberg K, Hagen A & Omholt SW (2004) Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite *Varroa destructor* (Acari: Varroidae): a factor in colony loss during overwintering? Journal of Economic Entomology 97: 741-747.

Anderson D & Trueman J (2000) *Varroa jacobsoni* (Acari: Varroidae) is more than one species. Experimental and Applied Acarology 24: 165-189.

Annoscia D, Del Piccolo F & Nazzi F (2012) How does the mite *Varroa destructor* kill the honeybee *Apis mellifera*? Alteration of cuticular hydrcarbons and water loss in infested honeybees. Journal of Insect Physiology 58: 1548-1555.

Arathi H, Burns I & Spivak M (2000) Ethology of hygienic behaviour in the honey bee *Apis mellifera* L.(Hymenoptera: Apidae): behavioural repertoire of hygienic bees. Ethology 106: 365-379.

Aumeier P & Rosenkranz P (2001) Scent or movement of *Varroa destructor* mites does not elicit hygienic behaviour by Africanized and Carniolan honey bees. Apidologie 32: 253-264.

Bertholf L (1925) The moults of the honeybee. Journal of Economic Entomology 18: 380-384.

Beshers SN & Fewell JH (2001) Models of division of labor in social insects. Annual Review of Entomology 46: 413-440.

Boecking O, Bienefeld K & Drescher W (2000) Heritability of the *Varroa*, a specific hygienic behaviour in honey bees (Hymenoptera: Apidae). Journal of Animal Breeding and Genetics 117: 417-424.

Boecking O & Drescher W (1992) The removal response of *Apis mellifera* L. colonies to brood in wax and plastic cells after artificial and natural infestation with *Varroa jacobsoni* Oud. and to freeze-killed brood. Experimental & Applied Acarology 16: 321-329.

Boutin S, Alburaki M, Mercier P-L, Giovenazzo P & Derome N (2015) Differential gene expression between hygienic and non-hygienic honeybee (*Apis mellifera* L.) hives. BMC Genomics 16: 500.

Bowen-Walker P, Martin S & Gunn A (1999) The Transmission of Deformed Wing Virus between Honeybees (*Apis mellifera* L.) by the Ectoparasitic Mite *Varroa jacobsoni* Oud. Journal of Invertebrate Pathology 73: 101-106.

Brian M (1975) Larval recognition by workers of the ant Myrmica. Animal Behaviour 23: 745-756.

Burley LM, Fell RD & Saacke RG (2008) Survival of honey bee (Hymenoptera: Apidae) spermatozoa incubated at room temperature from drones exposed to miticides. Journal of Economic Entomology 101: 1081-1087.

Carreck NL (2011) *Varroa*. Still a problem in the 21st century? International Bee Research Association.

Chen Y, Pettis JS, Evans JD, Kramer M & Feldlaufer MF (2004) Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. Apidologie 35: 441-448.

Chen YP & Siede R (2007) Honey bee viruses. Advances in Virus Research 70: 33-80.

Choe D-H, Millar JG & Rust MK (2009) Chemical signals associated with life inhibit necrophoresis in Argentine ants. Proceedings of the National Academy of Sciences 106: 8251-8255.

Chouvenc T, Robert A, Sémon E & Bordereau C (2012) Burial behaviour by dealates of the termite *Pseudacanthotermes spiniger* (Termitidae, Macrotermitinae) induced by chemical signals from termite corpses. Insectes Sociaux 59: 119-125.

Collins AM, Pettis JS, Wilbanks R & Feldlaufer MF (2004) Performance of honey bee (*Apis mellifera*) queens reared in beeswax cells impregnated with coumaphos. Journal of Apicultural Research 43: 128-134.

Cremer S, Armitage SAO & Schmid-Hempel P (2007) Social immunity. Current Biology 17: R693-R702.

D'Aubeterre JP, Myrold DD, Royce LA & Rossignol PA (1999) A scientific note of an application of isotope ratio mass spectrometry to feeding by the mite, *Varroa jacobsoni* Oudemans, on the honeybee, *Apis mellifera* L. Apidologie 30: 351-352.

Danka RG, Harris JW, Villa JD & Dodds GE (2013) Varying congruence of hygienic responses to *Varroa destructor* and freeze-killed brood among different types of honeybees. Apidologie 44: 447-457.

De Guzman LI & Rinderer TE (1999) Identification and comparison of *Varroa* species infesting honey bees. Apidologie 30: 85-95.

De Jong D, De Jong P & Goncalves L (1982) Weight loss and other damage to developing worker honeybees from infestation with *Varroa jacobsoni*. Journal of Apicultural Research 21: 165-167.

Dekeyser MA & Downer RGH (1994) Biochemical and physiological targets for miticides. Pesticide Science 40: 85-101.

Dietemann V, Nazzi F, Martin SJ, Anderson DL, Locke B, Delaplane KS, Wauquiez Q, Tannahill C, Frey E & Ziegelmann B (2013) Standard methods for *Varroa* research. Journal of Apicultural Research 52: 1.

Dietemann V, Pflugfelder J, Anderson D, Charrière J-D, Chejanovsky N, Dainat B, De Miranda JR, Delaplane K, Dillier F-X & Fuch S (2012) *Varroa destructor*: research avenues towards sustainable control. Journal of Apicultural Research 51: 125-132.

Espinosa-Montano LG-N, E; Sanchez-Albarrant, A; Montaldo, H.; Correa-Benitex, A. (2008) Comparative study of three assays to evaluate hygienic behavior in honey bee (Apis meiiifera L.) colonies. Veterinaria Mexico 39: 39-53.

Fakhimzadeh K (2001) Effectiveness of confectioner sugar dusting to knock down *Varroa destructor* from adult honey bees in laboratory trials. Apidologie 32: 139-148.

Fewell J & Page Jr R (1993) Genotypic variation in foraging responses to environmental stimuli by honey bees, *Apis mellifera*. Experientia 49: 1106-1112.

Feyereisen R (1999) Insect P450 enzymes. Annual Review of Entomology 44: 507-533.

Frazier M, Mullin C, Frazier J & Ashcraft S (2008) What have pesticides got to do with it? American Bee Journal 148: 521-524.

Free J & Spencer‐Booth Y (1959) The longevity of worker honey bees (*Apis mellifera*), Vol. 34: Proceedings of the Royal Entomological Society of London. Series A, General Entomology (ed. Wiley Online Library, pp. 141-150.

Free J & Winder M (1983) Brood recognition by honeybee (*Apis mellifera*) workers. Animal Behaviour 31: 539-545.

Frey E, Odemer R, Blum T & Rosenkranz P (2013) Activation and interruption of the reproduction of *Varroa destructor* is triggered by host signals (*Apis mellifera*). Journal of Invertebrate Pathology.

Garedew A, Schmolz E & Lamprecht I (2004) The energy and nutritional demand of the parasitic life of the mite *Varroa destructor*. Apidologie 35: 419-430.

Genersch E, Von Der Ohe W, Kaatz H, Schroeder A, Otten C, Büchler R, Berg S, Ritter W, Mühlen W & Gisder S (2010) The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41: 332-352.

Glancey BM, Stringer C, Craig C, Bishop P & Martin B (1970) Pheromone may induce brood tending in the fire ant, Solenopsis saevissima. Nature Vol. 226 pp. 863-864 pp.

Goode K, Huber Z, Mesce KA & Spivak M (2006) Hygienic behavior of the honey bee (*Apis mellifera*) is independent of sucrose responsiveness and foraging ontogeny. Hormones and Behavior 49: 391-397.

Gramacho KP & Spivak M (2003) Differences in olfactory sensitivity and behavioral responses among honey bees bred for hygienic behavior. Behavioral Ecology and Sociobiology 54: 472-479.

Haarmann T, Spivak M, Weaver D, Weaver B & Glenn T (2002) Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. Journal of Economic Entomology 95: 28-35.

Harbo JR & Harris JW (2001) Resistance to *Varroa destructor* (Mesostigmata: Varroidae) when mite-resistant queen honey bees (Hymenoptera: Apidae) were free-mated with unselected drones. Journal of Economic Entomology 94: 1319-1323.

Harbo JR & Harris JW (2005) Suppressed mite reproduction explained by the behaviour of adult bees. Journal of Apicultural Research 44: 21-23.

Harbo JR & Harris JW (2009) Responses to *Varroa* by honey bees with different levels of *Varroa* Sensitive Hygiene. Journal of Apicultural Research 48: 156.

Harris JW (2007) Bees with *Varroa* Sensitive Hygiene preferentially remove mite infested pupae aged ≤ five days post capping. Journal of Apicultural Research 46: 134-139.

Harris JW, Danka RG & Villa JD (2010) Honey bees (Hymenoptera: Apidae) with the trait of *Varroa* sensitive hygiene remove brood with all reproductive stages of *Varroa* mites (Mesostigmata: Varroidae). Annals of the Entomological Society of America 103: 146-152.

Harris JW, Danka RG & Villa JD (2012) Changes in infestation, cell cap condition, and reproductive status of *Varroa destructor* (Mesostigmata: Varroidae) in brood exposed to honey bees with *Varroa* sensitive hygiene. Annals of the Entomological Society of America 105: 512-518.

Herrmann M, Kanbar G & Engels W (2005) Survival of honey bee (*Apis mellifera*) pupae after trypan blue staining of wounds caused by *Varroa destructor* mites or artificial perforation. Apidologie 36: 107-111.

Holden C (2006) Report warns of looming pollination crisis in North America. Science 314: 397-397.

Ibrahim A, Reuter GS & Spivak M (2007) Field trial of honey bee colonies bred for mechanisms of resistance against *Varroa destructor*. Apidologie 38: 67-76.

Ibrahim A & Spivak M (2006) The relationship between hygienic behavior and suppression of mite reproduction as honey bee (*Apis mellifera*) mechanisms of resistance to *Varroa destructor*. Apidologie 37: 31.

Ifantidis MD (1988) Some aspects of the process of *Varroa jacobsoni* mite entrance into honey bee (*Apis mellifera*) brood cells. Apidologie 19: 387-396.

Imdorf AC, J D; Kilchemann, V; Bogdanov, S; Fluri, P (2003) Alternative strategy in central Europe for the control of *Varroa destructor* in honey bee colonies. Apiacta 38: 258-278.

Johnson RM, Ellis MD, Mullin CA & Frazier M (2010) Pesticides and honey bee toxicity, USA. Apidologie 41: 312-331.

Kanbar G & Engels W (2003) Ultrastructure and bacterial infection of wounds in honey bee (*Apis mellifera*) pupae punctured by *Varroa* mites. Parasitology Research 90: 349-354.

Kather R, Drijfhout FP, Shemilt S & Martin SJ (2015) Evidence for passive chemical camouflage in the parasitic mite *Varroa destructor*. Journal of Chemical Ecology 41: 178-186.

Kearns CA, Inouye DW & Waser NM (1998) Endangered mutualisms: the conservation of plant-pollinator interactions. Annual Review of Ecology and Systematics: 83-112.

Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C & Tscharntke T (2007) Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences 274: 303-313.

Kuster RD, Boncristiani HF & Rueppell O (2014) Immunogene and viral transcript dynamics during parasitic *Varroa destructor* mite infection of developing honey bee (*Apis mellifera*) pupae. The Journal of Experimental Biology 217: 1710-1718.

Le Conte Y, Alaux C, Martin JF, Harbo J, Harris J, Dantec C, Severac D, Cros‐Arteil S & Navajas M (2011) Social immunity in honeybees (*Apis mellifera*): transcriptome analysis of *Varroa*‐hygienic behaviour. Insect Molecular Biology 20: 399-408.

Le Conte Y, Arnold G, Trouiller J, Masson C & Chappe B (1990) Identification of a brood pheromone in honeybees. Naturwissenschaften 77: 334-336.

Le Conte Y, Huang Z, Roux M, Zeng Z, Christidès J-P & Bagnères A-G (2015) *Varroa destructor* changes its cuticular hydrocarbons to mimic new hosts. Biology Letters 11: 20150233.

Le Conte Y, Sreng L & Poitout SH (1995) Brood Pheromone Can Modulate the Feeding Behavior of *Apis mellifera* Workers (Hytnenoptera: Apidae). Journal of Economic Entomology 88: 798-804.

Lee KV, Steinhauer N, Rennich K, Wilson ME, Tarpy DR, Caron DM, Rose R, Delaplane KS, Baylis K & Lengerich EJ (2015) A national survey of managed honey bee 2013–2014 annual colony losses in the USA. Apidologie 46: 292-305.

Martin C, Provost E, Bagnères AG, Roux M, Clément JL & Le Conte Y (2002) Potential mechanism for detection by *Apis mellifera* of the parasitic mite *Varroa destructor* inside sealed brood cells. Physiological Entomology 27: 175-188.

Martin C, Salvy M, Provost E, Bagnères A-G, Roux M, Crauser D, Clement J-L & Le Conte Y (2001) Variations in chemical mimicry by the ectoparasitic mite *Varroa jacobsoni* according to the developmental stage of the host honey-bee *Apis mellifera*. Insect Biochemistry and Molecular Biology 31: 365-379.

Martin SJ, Highfield AC, Brettell L, Villalobos EM, Budge GE, Powell M, Nikaido S & Schroeder DC (2012) Global Honey Bee Viral Landscape Altered by a Parasitic Mite. Science 336: 1304-1306.

Masterman R, Ross R, Mesce K & Spivak M (2001) Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L.). Journal of Comparative Physiology A 187: 441-452.

Masterman R, Smith B & Spivak M (2000) Brood odor discrimination abilities in hygienic honey bees (*Apis mellifera* L.) using proboscis extension reflex conditioning. Journal of Insect Behavior 13: 87-101.

Maurizio A & Hodges F (1950) The influence of pollen feeding and brood rearing on the length of life and physiological condition of the honeybee preliminary report. Bee World 31: 9-12.

Meixner MD (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology 103: S80-S95.

Memmott J, Waser NM & Price MV (2004) Tolerance of pollination networks to species extinctions. Proceedings of the Royal Society of London. Series B: Biological Sciences 271: 2605-2611.

Monnin T & Peeters C (1997) Cannibalism of subordinates' eggs in the monogynous queenless ant Dinoponera quadriceps. Naturwissenschaften 84: 499-502.

Navajas M, Migeon A, Alaux C, Martin-Magniette M-L, Robinson G, Evans J, Cros-Arteil S, Crauser D & Le Conte Y (2008) Differential gene expression of the honey bee *Apis mellifera* associated with *Varroa destructor* infection. BMC Genomics 9: 301.

Nazzi F, Brown SP, Annoscia D, Del Piccolo F, Di Prisco G, Varricchio P, Della Vedova G, Cattonaro F, Caprio E & Pennacchio F (2012) Synergistic Parasite-Pathogen Interactions Mediated by Host Immunity Can Drive the Collapse of Honeybee Colonies. PLoS Pathogens 8: e1002735.

Parker R, Guarna MM, Melathopoulos AP, Moon K-M, White R, Huxter E, Pernal SF & Foster LJ (2012) Correlation of proteome-wide changes with social immunity behaviors provides insight into resistance to the parasitic mite, *Varroa destructor*, in the honey bee (*Apis mellifera*). Genome Biology 13: R81.

Pernal SF, Sewalem A & Melathopoulos AP (2012) Breeding for hygienic behaviour in honeybees (*Apis mellifera*) using free-mated nucleus colonies. Apidologie: 1-14.

Pettis J, Shimanuki WWH & Teel P (1991) Fluvalinate treatment of queen and worker honey bees (*Apis mellifera L*) and effects on subsequent mortality, queen acceptance and supersedure. Apidologie 22: 1-7.

Pettis JS, Collins AM, Wilbanks R & Feldlaufer MF (2004) Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. Apidologie 35: 605-610.

Pettis JS & Delaplane KS (2010) Coordinated responses to honey bee decline in the USA. Apidologie 41: 256-263.

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O & Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. Trends in Ecology & Evolution 25: 345-353.

Rinderer TE, Harris JW, Hunt GJ & De Guzman LI (2010) Breeding for resistance to *Varroa destructor* in North America. Apidologie 41: 409-424.

Rosenkranz P, Aumeier P & Ziegelmann B (2010) Biology and control of *Varroa destructor*. Journal of Invertebrate Pathology 103: S96-S119.

Salvy M, Martin C, Bagneres A, Provost E, Roux M, Le Conte Y & Clement J (2001) Modifications of the cuticular hydrocarbon profile of *Apis mellifera* worker bees in the presence of the ectoparasitic mite *Varroa jacobsoni* in brood cells. Parasitology 122: 145-159.

Sammataro D, Untalan P, Guerrero F & Finley J (2005) The resistance of *Varroa* mites (Acari: Varroidae) to acaricides and the presence of esterase. International Journal of Acarology 31: 67-74.

Santomauro G, Oldham NJ, Boland W & Engels W (2004) Cannibalism of diploid drone larvae in the honey bee (*Apis mellifera*) is released by odd pattern of cuticular substances. Journal of Apicultural Research 43: 69-74.

Schatton-Gadelmayer K & Engels W (1988) Blood proteins and body weight of newly-emerged worker honeybees with different levels of parasitization of brood mites. Entomolgia Generalis 14: 93-101.

Schöning C, Gisder S, Geiselhardt S, Kretschmann I, Bienefeld K, Hilker M & Genersch E (2012) Evidence for damage-dependent hygienic behaviour towards *Varroa destructor*-parasitised brood in the western honey bee, *Apis mellifera*. The Journal of Experimental Biology 215: 264-271.

Spivak M (1996) Honey bee hygienic behavior and defense against *Varroa jacobsoni*. Apidologie 27: 245-260.

Spivak M & Downey DL (1998) Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). Journal of Economic Entomology 91: 64-70.

Spivak M & Gilliam M (1998) Hygienic behaviour of honey bees and its application for control of brood diseases and *Varroa*: Part II. Studies on hygienic behaviour since the Rothenbuhler era. Bee World 79: 169-186.

Spivak M, Masterman R, Ross R & Mesce KA (2003) Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. Journal of Neurobiology 55: 341-354.

Spivak M & Reuter GS (2001a) Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. Apidologie 32: 555-565.

Spivak M & Reuter GS (2001b) *Varroa destructor* infestation in untreated honey bee (Hymenoptera: Apidae) colonies selected for hygienic behavior. Journal of Economic Entomology 94: 326-331.

Spleen AM, Lengerich EJ, Rennich K, Caron D, Rose R, Pettis JS, Henson M, Wilkes JT, Wilson M & Stitzinger J (2013) A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. Journal of Apicultural Research 52: 44-53.

Spotter A, Gupta P, Nurnberg G, Reinsch N & Bienefeld K (2012) Development of a 44K SNP assay focusing on the analysis of a *Varroa*-specific defence behaviour in honey bees (Apis mellifera carnica). Molecular Ecology Resources.

Steinhauer N, Rennich K, Lee KV, Pettis J, Tarpy DR, Rangel J, Caron D, Sagili R, Skinner JA, Wilson ME, Wilkes JT, Delaplane KS, Rose R & van Engelsdorp D (2015) Colony Loss 2014-2015: Preliminary Results, Vol. 2015.

Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS, Rose R, Skinner JA, Tarpy DR & Wilkes JT (2014) A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. Journal of Apicultural Research 53: 1-18.

Sun Q & Zhou X (2013) Corpse management in social insects. International Journal of Biological Sciences 9: 313.

Swanson JA, Torto B, Kells SA, Mesce KA, Tumlinson JH & Spivak M (2009) Odorants that induce hygienic behavior in honeybees: identification of volatile compounds in chalkbrood-infected honeybee larvae. Journal of Chemical Ecology 35: 1108-1116.

Sylvester HA, Watts RP, Guzman LI, Stelzer JA & Rinderer TE (1999) *Varroa* in the mating yard. II. The effects of *Varroa* and fluvalinate on drone mating competitiveness. American Bee Journal 139.

Toufailia HMA, Amiri E, Scandian L, Kryger P & Ratnieks FL (2014) Towards integrated control of *Varroa*: effect of variation in hygienic behaviour among honey bee colonies on mite population increase and deformed wing virus incidence. Journal of Apicultural Research 53: 555-562.

Traynor KS, Le Conte Y & Page RE (2015) Age matters: pheromone profiles of larvae differentially influence foraging behaviour in the honeybee, *Apis mellifera*. Animal Behaviour 99: 1-8.

Tsuruda JM, Harris JW, Bourgeois L, Danka RG & Hunt GJ (2012) High-resolution linkage analyses to identify genes that influence *Varroa* sensitive hygiene behavior in honey bees. PLoS ONE 7: e48276.

Ulyshen MD & Shelton TG (2012) Evidence of cue synergism in termite corpse response behavior. Naturwissenschaften 99: 89-93.

vanEngelsdorp D, Caron D, Hayes J, Underwood R, Henson M, Rennich K, Spleen A, Andree M, Snyder R & Lee K (2012) A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. Journal of Apicultural Research 51: 115-124.

Walsh JP & Tschinkel W (1974) Brood recognition by contact pheromone in the red imported fire ant, Solenopsis invicta. Animal Behaviour 22: 695-704.

Watkins J & Cole T (1966) Attraction of army ant workers to secretions of their queens. Texas Journal of Science 18: 254.

Wheeler WM (1910) Ants: their structure, development and behavior. Columbia University Press, New York.

Wilson-Rich N, Spivak M, Fefferman NH & Starks PT (2009) Genetic, individual, and group facilitation of disease resistance in insect societies. Annual Review of Entomology 54: 405-423.

Yang X & Cox-Foster DL (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proceedings of the National Academy of Sciences of the United States of America 102: 7470-7475.

Tables

Table 1. Statistically Significant Factors and Interactions from a Full Factorial Logistic Regression Model on Colony Type, Brood Type, Treatment and Year.

Factor	Wald	d.f.	Sig.	EXP(B)	95% C.I. for EXP(B)	
					Lower	Upper
VSH Colony	5.436	1	0.020	0.072	0.008	0.658
HYG Brood	6.699	1	0.010	0.162	0.041	0.643
VSH Brood	7.165	1	0.007	0.098	0.018	0.537
Year	10.914	1	0.001	0.025	0.003	0.225
HYG Brood x VSH Colony	8.739	1	0.003	44.045	3.581	541.807
VSH Brood x VSH Colony	4.365	1	0.037	20.364	1.205	344.068
HYG Brood x Mite Treatment	4.996	1	0.025	7.771	1.287	46.922
VSH Brood x Year	8.482	1	0.004	64.684	3.910	1,070.046
VSH Brood x VSH Colony x Year	4.211	1	0.040	0.008	<0.001	0.807

Table 2. Percent Hygiene Levels for Individual Colonies, as Determined by Freeze-Killed Brood Assays.

Colony Type	Year	% Hygienic (FKB Assay)
CON	2013	51
HYG	2013	91
HYG	2013	99
HYG	2013	100
VSH	2013	46
VSH	2013	80
CON	2014	48
HYG	2014	95
HYG	2014	99
VSH	2014	100
VSH	2014	97

Figures

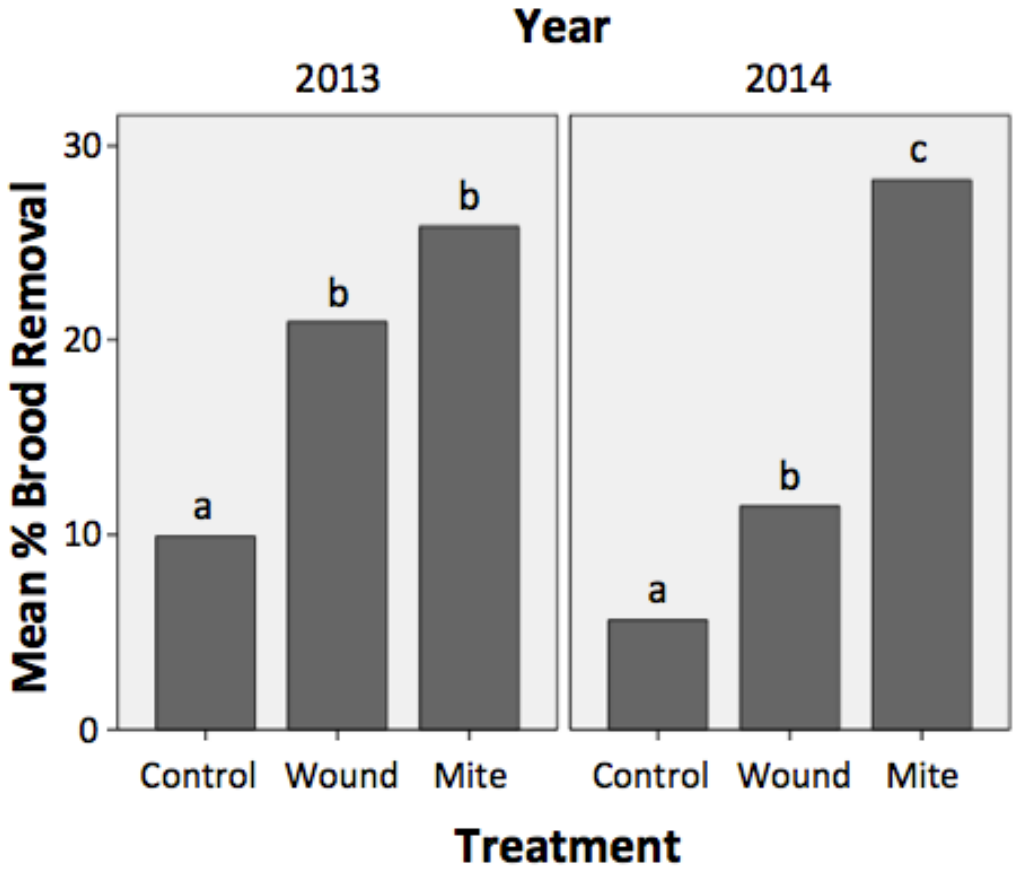

Figure 1. Treatment Effects on Mean Percent Brood Removal, Irrespective of Brood or Colony Type. Different letters indicate significant differences from Chi-square analysis ($p<0.05$). In 2013, removal of mite-infested brood by all colonies, irrespective of type, was not significantly higher than removal of wounded brood ($\chi^2=2.4$, d.f.=1, $p=0.125$) but was significantly higher than removal of control brood ($\chi^2=31.2$, d.f.=1, $p<0.001$). Removal of wounded brood was significantly higher than removal of control brood in 2013 ($\chi^2=16.6$, d.f.=1, $p<0.001$). In 2014 removal of mite-infested brood was significantly higher than removal of wounded brood ($\chi^2=30.3$, d.f.=1, $p<0.001$) and control brood ($\chi^2=61.9$, d.f.=1, $p<0.001$). Removal of wounded brood was significantly higher than removal of control brood in 2014 ($\chi^2=7.4$, d.f.=1, $p=0.006$). Total sample sizes were 701, 684, and 703 for control, wound and mite treatments, respectively.

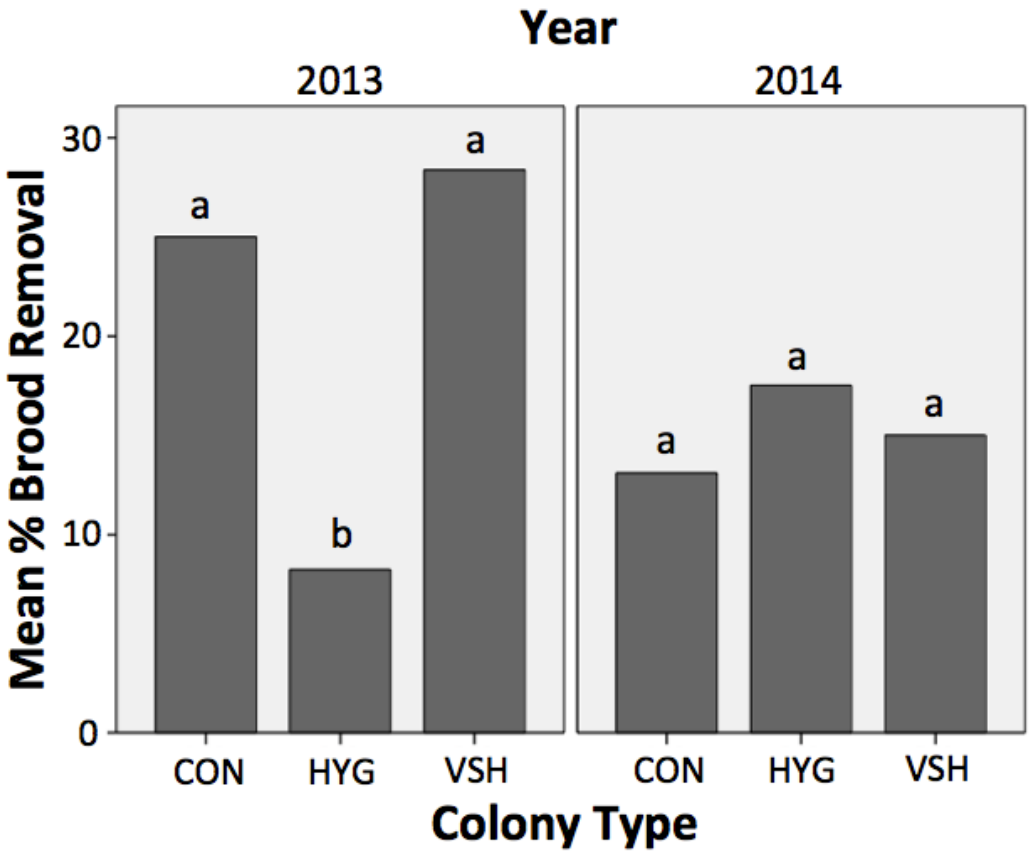

Figure 2. Colony Type Effects on Mean Percent Brood Removal, Irrespective of Brood or Treatment Type. Different letters indicate significant differences from Chi-square analysis ($p<0.05$). In 2013, removal of brood from all treatment groups by bees in VSH colonies was significantly higher than removal of brood by bees in HYG colonies ($\chi^2=57.5$, d.f.=1, $p<0.001$), but not significantly different than removal of brood by bees in CON colonies ($\chi^2=0.8$, d.f.=1, $p=0.359$). Removal of brood by bees in HYG colonies was significantly lower than removal of brood by bees in CON colonies in 2013 ($\chi^2=37.8$, d.f.=1, $p<0.001$). In 2014, there was no significant difference between removal by bees in CON and HYG colonies ($\chi^2=2.6$, d.f.=1, $p=0.104$), CON and VSH colonies ($\chi^2=0.5$, d.f.=1, $p=0.480$), or HYG and VSH colonies ($\chi^2=0.8$, d.f.=1, $p=0.378$). Total sample sizes were 603, 816, and 669 for CON, HYG and VSH colonies, respectively.

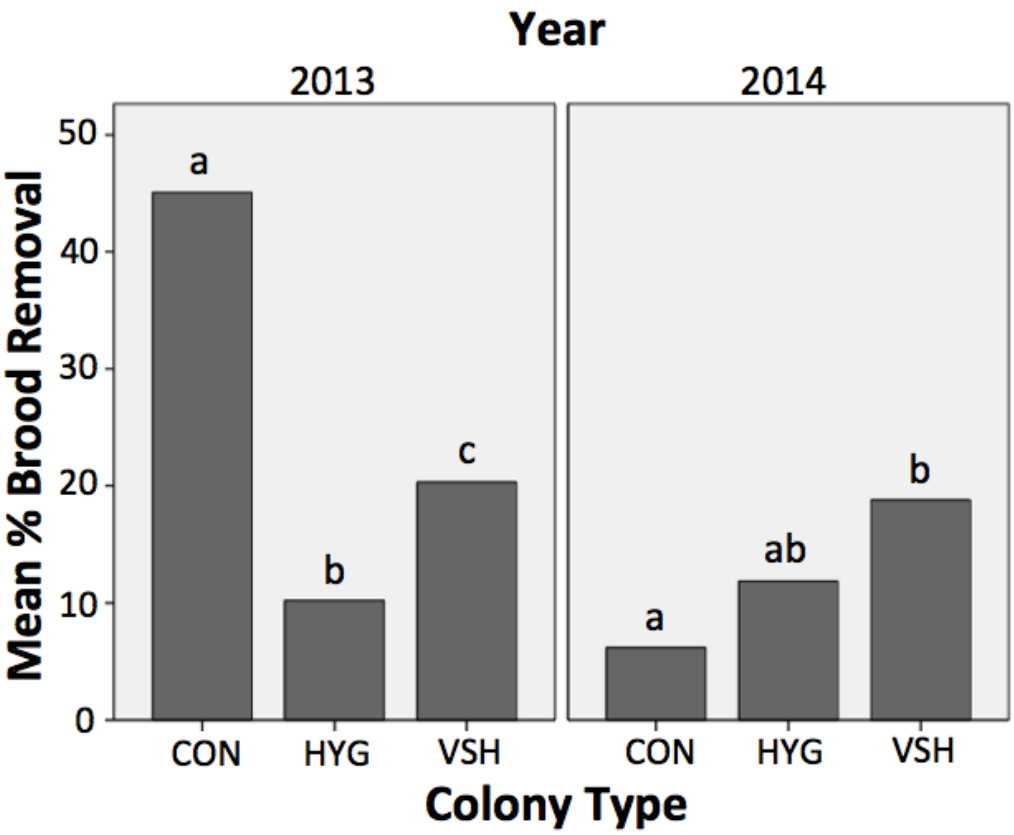

Figure 3. Colony Type Effects on Mean Percent Brood Removal, Irrespective of Treatment Type, for the Subset of Data in which Colony Type was the Same as Brood Type. Different letters indicate significant differences from Chi-square analysis ($p<0.05$). In 2013, removal of brood by bees in VSH colonies was significantly higher than removal of brood by bees in HYG colonies ($\chi^2=5.26$, d.f.=1, $p=0.022$), and significantly lower than removal of brood by bees in CON colonies ($\chi^2=11.269$, d.f.=1, $p=0.001$). Removal of brood by bees in HYG colonies was significantly lower than removal of brood by bees in CON colonies in 2013 ($\chi^2=28.602$, d.f.=1, $p<0.001$). In 2014, removal of brood by bees in VSH colonies was significantly higher than removal of brood by bees in CON colonies ($\chi^2=10.548$, d.f.=1, $p=0.001$), but was not significantly different from removal of brood by bees in HYG colonies ($\chi^2=1.973$, d.f.=1, $p=0.160$). Removal of brood by bees in HYG colonies was not significantly different than removal of brood by bees in CON colonies in 2014 ($\chi^2=2.594$, d.f.=1, $p=0.107$). Total sample sizes were 212, 238, and 245 for CON, HYG and VSH colonies, respectively.

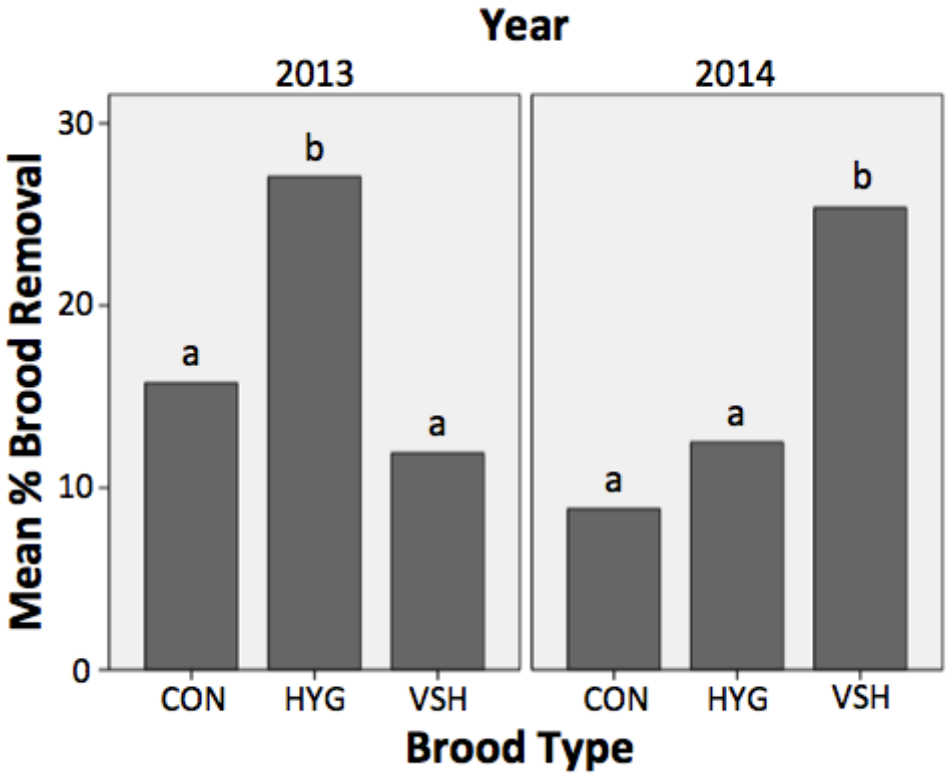

Figure 4. Brood Type Effects on Mean Percent Brood Removal, Irrespective of Colony or Treatment Type. Different letters indicate significant differences from Chi-square analysis ($p<0.05$). In 2013, removal of VSH brood was significantly lower than removal of HYG brood ($\chi^2=27.6$, d.f.=1, $p<0.001$) but not significantly different than removal of CON brood ($\chi^2=2.0$, d.f.=1, $p=0.155$). Removal of HYG brood was significantly higher than removal of CON brood in 2013 ($\chi^2=12.9$, d.f.=1, $p<0.001$). In 2014, removal of VSH brood was significantly higher than removal of HYG brood ($\chi^2=15.2$, d.f.=1, $p<0.001$) and CON brood ($\chi^2=38.3$, d.f.=1, $p<0.001$). Removal of HYG brood was not significantly different than removal of CON brood in 2014 ($\chi^2=2.3$, d.f.=1, $p=0.125$). Total sample sizes were 722, 666, and 700 for CON, HYG and VSH brood, respectively.

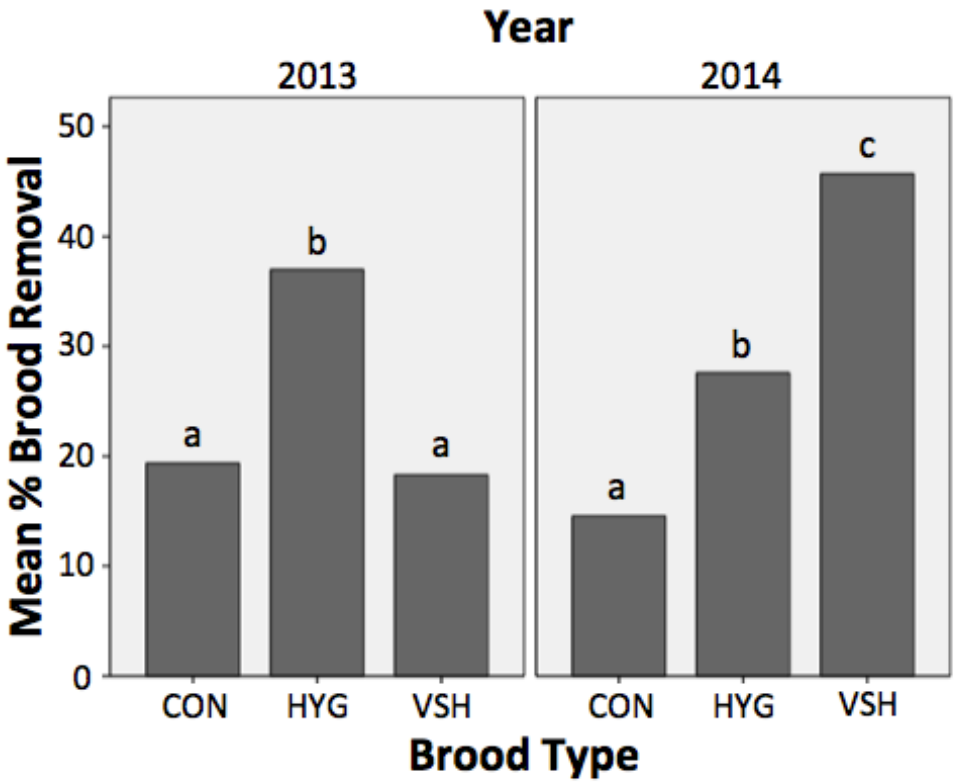

Figure 5. Brood Type Effects on Mean Percent Brood Removal for Mite-Infested Cells Only. Different letters indicate significant differences from Chi-square analysis ($p<0.05$). In 2013, removal of VSH brood was significantly lower than removal of HYG brood ($\chi^2=11.0$, d.f.=1, $p=0.001$) but not significantly different than removal of CON brood ($\chi^2=0.0$, d.f.=1, $p=0.843$). Removal of HYG brood was significantly higher than removal of CON brood in 2013 ($\chi^2=8.5$, d.f.=1, $p=0.004$). In 2014, removal of VSH brood was significantly higher than removal of HYG brood ($\chi^2=6.9$, d.f.=1, $p=0.009$) and CON brood ($\chi^2=30.5$, d.f.=1, $p<0.001$). Removal of HYG brood was significantly higher than removal of CON brood in 2014 ($\chi^2=8.5$, d.f.=1, $p=0.004$). Total sample sizes were 242, 225, and 236 for CON, HYG and VSH brood, respectively.

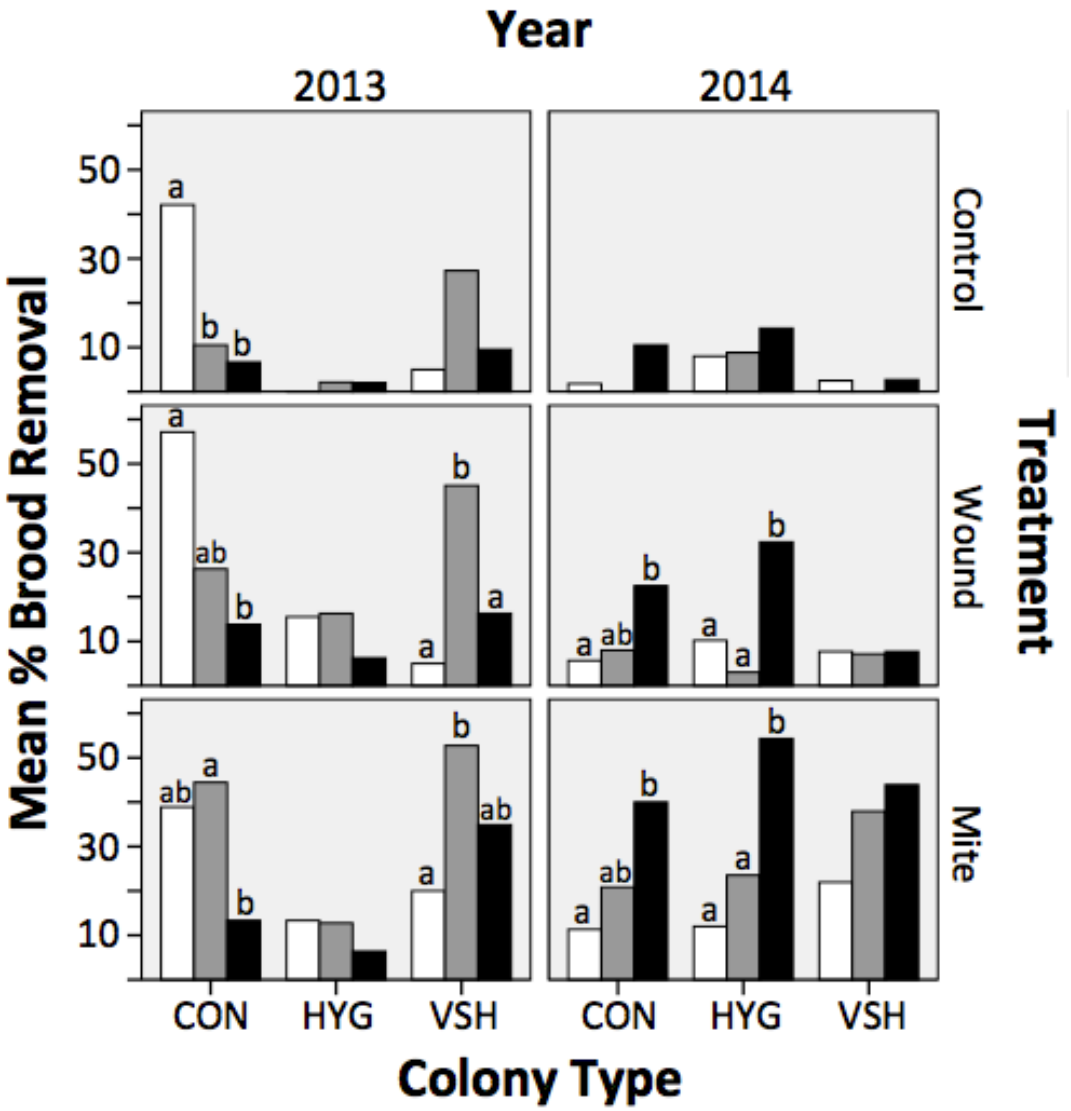

Figure 6. Treatment, Colony Type and Brood Type Effects on Mean Percent Brood Removal. White, gray and black bars represent CON, HYG and VSH brood, respectively. Different letters indicate significant differences from Chi-square analysis after Bonferroni correction ($p<0.0167$) within each treatment by colony type by year combination.

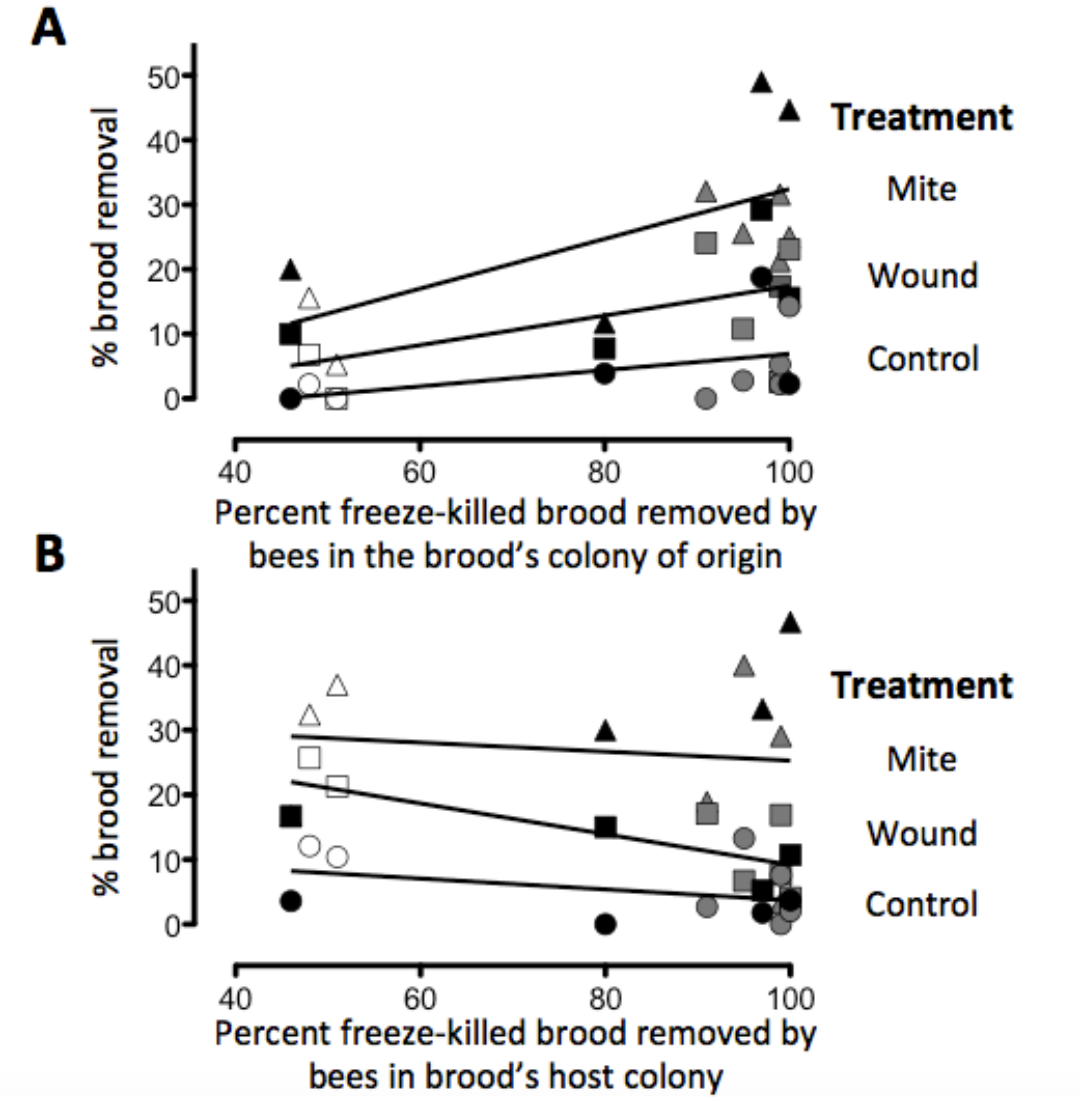

Figure 7. A) Percent Brood Removal Plotted Against the Percent of Freeze-Killed Brood Removed by Bees in the Brood's Colony of Origin and B) Percent Brood Removal Plotted Against Mean Percent of Freeze-Killed Brood Removed by Bees in the Brood's Host Colony. Triangular, square, and circular markers represent mite, wound, and control treatments, and correspond to the lines of best fit drawn beside respective treatment labels. White, gray and black symbols represent CON, HYG and VSH colonies, respectively. Colonies without percent freeze-killed brood data (n=4) are not included. A significant positive correlation was observed for mite-infested brood when considering hygienic level of the brood's colony of origin. A significant negative correlation was observed for wounded brood when considering hygienic level of the brood's host colony.

CHAPTER III

ASSOCIATION OF CHEMICAL BROOD SIGNALS OF THE HONEY BEE (*APIS MELLIFERA*) WITH HYGIENIC BEHAVIOR AND RELATED STRESSORS

This chapter is coauthored by Kaira M. Wagoner, Daniel C. Smith, Marla Spivak, Abraham Hefetz, and Olav Rueppell.

Introduction

Despite an increasing demand for crop pollinators (Aizen et al., 2008; Aizen & Harder, 2009), the health of the honey bee *(Apis mellifera)* is declining worldwide (Council, 2006; Genersch et al., 2010; Potts et al., 2010; vanEnglesdorp et al., 2008). In the United States, the total annual losses of managed honey bee colonies has exceeded 33% in all but one of the last five years (Lee et al., 2015; Spleen et al., 2013; Steinhauer et al., 2015; Steinhauer et al., 2014; vanEngelsdorp et al., 2012). This honey bee health crisis is of grave economic concern, as honey bee crop pollination has an estimated $16.4 billion-dollar effect on annual crop yield in the United States alone (Losey & Vaughan, 2006; Morse & Calderone, 2000) and globally, honey bees are the most important pollinator of large-scale agricultural crops (Genersch et al., 2010; Morse & Calderone, 2000; Williams, 1994). Furthermore, in light of recent declines in native pollinators (Biesmeijer et al., 2006; Grixti et al., 2009; Potts et al., 2010), honey bees have an increasingly important pollination function in natural ecosystems, and therefore contribute significantly to environmental health (Memmott et al., 2004; Potts et al., 2010).

Recent declines in honey bee health are attributed primarily to the introduction and spread of parasites and their associated pathogens (Nazzi et al., 2012; Potts et al., 2010). The obligate, ectoparasitic honey bee mite *Varroa destructor* (*Varroa*) may be the most important threat to honey bee health today (Anderson & Trueman, 2000; Rosenkranz et al., 2010). *Varroa* was originally a parasite of the Asian honey bee, *Apis cerana.* Around four decades ago, *Varroa* made a host-shift to the European honey bee *Apis mellifera* (Anderson & Trueman, 2000), and the mite was first observed in North America in 1987 (Wenner & Bushing, 1996). *Varroa* mites require access to honey bee larvae for reproduction. In order to reproduce, foundress mites enter the cells of uncapped, 5^{th} larval instars and conceal themselves in the food stored at the base of the cell. After the food has been consumed by the honey bee brood, the mite emerges and begins to feed on the brood hemolymph (Ifantidis, 1988). The mite lays her first egg around 70 hours after capping of the honey bee cell. The first egg is haploid and develops into a male (Rosenkranz et al., 2010). Deposition of the single haploid egg is followed by deposition of diploid eggs at approximately 30-hour intervals. Diploid eggs develop into females, which then mate with their waiting brother such that as many as four (worker brood cell) or five (drone brood cell) fertilized female *Varroa* may emerge with the adult honey bee (Rosenkranz et al., 2010). The sucking of hemolymph by *Varroa* is harmful to honey bee health, and often leads to a decrease in body weight and protein levels (Amdam et al., 2004; D'Aubeterre et al., 1999; De Jong et al., 1982; Garedew et al., 2004; Schatton-Gadelmayer & Engel, 1988).

However, *Varroa* are more than a physical burden to the honey bee, as they also vector pathogens (Bowen-Walker et al., 1999; Chen et al., 2004; Kanbar & Engels, 2003; Martin et al., 2012), and have been associated with the amplification and increased susceptibility of honey bees to viruses (Bowen-Walker et al., 1999; Gisder et al., 2009; Martin et al., 2012; Yang & Cox-Foster, 2005). Deformed wing virus (Topolska et al., 1995) is a positive, single stranded RNA virus (Bailey & Ball, 1991) associated with deformed wings, a shortened abdomen, reduced weight, discoloration, and premature death (Boecking & Genersch, 2008; De Jong et al., 1982; De Miranda & Genersch, 2010). While deformed wing virus (DWV) can occur in the absence of *Varroa,* it is typically asymptomatic in such cases (Bowen-Walker et al., 1999; De Miranda & Genersch, 2010; Gisder et al., 2009; Ryabov et al., 2014). In contrast, DWV that is associated with *Varroa* is a significant contributor to honey bee health decline (De Miranda & Genersch, 2010; Highfield et al., 2009; Martin, 2001; Möckel et al., 2011; Nazzi et al., 2012; Yang & Cox-Foster, 2007).

Honey bees are especially susceptible to stressors like *Varroa* and DWV due to frequent contact with genetically similar nestmates (Cremer et al., 2007). As social insects, honey bees complement individual immunity with mechanisms of social immunity for defense against parasites and pathogens. Honey bees are able to reduce the loads of parasites and pathogens, such as *Varroa* and its associated viruses, through the age-specific sanitary activity of hygienic behavior. Hygienic behavior is the detection, uncapping and removal of diseased brood from the hive (Spivak & Reuter, 2001a; Wilson-Rich et al., 2009), and is most commonly observed in worker bees aged 15 to 20

days (Spivak et al., 2003). Due to the economic importance of healthy pollinators, breeding programs have enhanced *Varroa* resistance in some honey bees through positive selection for hygienic behavior. Two such hygienic colony-types are the Minnesota Hygienic (HYG), selected for based on the removal of freeze-killed brood (Spivak, 1996), and the *Varroa* Sensitive Hygienic (VSH), selected for based on apparent suppression of mite reproduction. Both HYG and VSH colonies have been shown repeatedly to exhibit reduced mite loads compared to unselected colonies (Harbo & Harris, 2001; Harris, 2007; Spivak & Reuter, 2001a).

However, despite these successes, interventions such as the use of miticides are still needed to control severe mite infestations in hygienic colonies (Ibrahim et al., 2007; Spivak & Reuter, 2001b). Miticides are harmful to honey bee health (Collins et al., 2004; Haarmann et al., 2002; Pettis et al., 1991; Pettis et al., 2004; Sylvester et al., 1999), and lack sustainability, as resistance to the chemicals often builds in mite populations over time (Rosenkranz et al., 2010; Sammataro et al., 2005). One sustainable solution for enhanced *Varroa* control would be to increase the resistance of hygienic colonies to mites through improved selective breeding methods (Boecking et al., 2000; Dietemann et al., 2012; Rinderer et al., 2010; Tsuruda et al., 2012), especially breeding methods that target mites and other common threats to honey bee health. However, to date, little is known about the olfactory trigger for hygienic behavior.

The role of heightened olfactory sensitivity of adult bees on enhanced removal rates by hygienic colonies has been established (Gramacho & Spivak, 2003; Masterman et al., 2001) and is supported by evidence that the neuromodulator octopamine has

greater immunoreactivity in neurons of bees performing hygienic activities, and that octopamine enhances sensitivity of non-hygienic bees to diseased brood odor (Goode et al., 2006; Spivak et al., 2003). However, despite these insights regarding adult perception, the source and physical nature of the trigger for hygienic behavior have remained unclear. It is apparent that foundress mites and their offspring play a role in triggering hygienic behavior (Harbo & Harris, 2009), however mites are known to mimic cuticular chemical profiles of brood (Le Conte et al., 2015; Martin et al., 2001; Nation et al., 1992), which likely makes them difficult to detect directly. Furthermore, hygienic behavior occurs in response to brood death and a variety of diseases (Gilliam et al., 1983; Gilliam et al., 1988; Schöning et al., 2012; Spivak & Reuter, 2001a), not all of which are linked to *Varroa,* suggesting that the source of the trigger for hygienic behavior may be the stressed brood rather than the stressors themselves. This is consistent with evidence for higher removal rates of mite-infested worker brood than mite-infested drone brood (Harris, 2008), despite a higher number of mite offspring typically being produced in drone brood (Rosenkranz et al., 2010; Rosenkranz & Renz, 2003). The notion that the trigger for hygienic behavior is a chemical signal that originates from the brood is also supported by our findings in cross-fostered bees that 1) removal rates differ by brood type despite similarities in levels of hygiene and 2) regardless of host colony, brood originating from colonies considered highly hygienic is removed more readily than brood originating from less hygienic colonies (see Chapter II).

Though fatty acids, monoterpenoids and hydrocarbons are all chemicals used for communication among insects (Blomquist & Vogt, 2003), cuticles of honey bees and

other hymenoptera are typically dominated by hydrocarbons, specifically alkanes, alkenes, and methylalkanes (Annoscia et al., 2012; Aumeier et al., 2002; Ayasse et al., 1999; Ferreira-Caliman et al., 2012; Francis et al., 1989; Francis et al., 1985). In insects, hydrocarbons are produced in oenocytes (Romer, 1991), specialized ectodermic cells found in the insect fat body. The biosynthesis of hydrocarbons in oenocytes includes elongation of fatty acids followed by the reduction of long chain fatty acids to aldehydes, and conversion of aldehydes to hydrocarbons through the loss of the carboxyl carbon (Blomquist & Vogt, 2003). Hydrocarbons are transferred via hemolymph from the oenocyte to the insect cuticles (Soroker & Hefetz, 2000), where they function both to prevent desiccation and facilitate communication (Annoscia et al., 2012; Howard, 1993; Howard & Blomquist, 2005; Lockey, 1988). When used for communication, hydrocarbon type and quantity can be discriminated with varying degrees of sensitivity for inter- and intra-colonial recognition tasks. For example, larger or more easily detected differences in hydrocarbon profiles may be required to discriminate nestmates from intruders (Dani et al., 2001; LeConte & Hefetz, 2008; Nascimento & Nascimento, 2012), while more discrete differences, perhaps differences in the relative quantities of single compounds, may be sufficient to discriminate intra-colonial age and caste designations (LeConte & Hefetz, 2008). In honey bees, for example, foragers from different colonies can be discriminated by a number of compounds, including alkanes, alkenes and alkadienes (Nascimento & Nascimento, 2012), and 4^{th} larval instars can be distinguished from 5^{th} larval instars by methylalkanes and alkenes (Aumeier et al., 2002).

Past studies have provided evidence of significant quantitative differences between hydrocarbons and other pheromones associated with brood parasitized by *Varroa* containing high and low levels of DWV (Schöning et al., 2012) and of *Varroa*-infested and non-infested brood (Salvy et al., 2001). The former study found that larvae associated with high-DWV mites was more likely to be removed, and could be characterized by a higher quantity of headspace hydrocarbons. The later study reported that larvae parasitized by one or more *Varroa* could be characterized by the 31- and 33-carbon alkenes hentriacontene and tritriacontene, respectively (Salvy et al., 2001). These findings support the notion that *Varroa*-induced differences in cuticular chemical profiles lead to damage-dependent removal. However, of these studies, only the later consistently correlated *specific* cuticular chemicals with a stressor, and results from this study have never been confirmed or explored in greater depth. Furthermore, no study has definitively linked stress-induced chemical differences to hygienic behavior, or explored whether variation in the nature and strength of stress-induced chemical signals plays a role in differentiating hygienic behavior between honey bee brood types. To address these knowledge gaps, we tested the hypothesis that hygienic behavior and related stressors are associated with chemical brood signals specific to honey bee brood type. More specifically, we predicted that 1) honey bee stressors affect chemical brood signals, 2) affects of honey bee stressors on chemical brood signals are specific to honey bee type, and 3) honey bee stressors that affect chemical brood signals are associated with hygienic behavior. We tested this hypothesis by performing two experiments. The first experiment compares chemical brood profiles from various colony types exposed to *Varroa* (and

consequently DWV) to address predictions 1 and 2. The second experiment compares cuticular hydrocarbon profiles from capped and hygienically uncapped brood cells to address prediction 3. Results from this study reveal the relationships between honey bee stressors, brood types, and brood chemical profiles, and link a specific chemical to cell uncapping, the first step in the process of hygienic behavior.

Materials and Methods

Experiment I: Effects of Treatment and Brood Type on Honey Bee Chemical Profiles

Sample Collection

This study was conducted over the summers of 2012, 2013 and 2014 to analyze how cuticular hydrocarbon profiles of *Varroa*-Sensitive Hygienic (VSH), Minnesota Hygienic (HYG) and unselected control (CON) honey bee brood are influenced by *Varroa* mites compared to wounding and control treatments. All sample collection and analysis was conducted at the University of North Carolina at Greensboro. Queens of VSH (n=6, 2 each in 2012, 2013 and 2014), HYG (n=8, 4 in 2012, and 2 each in 2013 and 2014), and CON (n=6, 4 in 2012 and 2 in 2014) origin were caged on wax foundation frames (1 queen per frame). All frames were drawn out in CON colonies immediately before the experiment. In 2013, CON queens did not perform as expected and therefore no data are available from that year for this group. Queen cages were removed from frames once eggs were laid in more than 75% of cells. After allowing 5 to 6 days for larval development, the locations of uncapped cells containing 5th instar larvae were marked using a permanent marker and transparent plastic sheets held in place above the

experimental cells with thumb tacks. Frames were placed back in the hives for no more than 16 hours to be capped. These recently capped cells were used to ensure that the experimental treatments were all applied to larvae of the same age, within 16 hours of capping. This time window is critical for successful initiation of mite oogenesis (Frey et al., 2013).

Within between 12 and 16 hours of cell capping, mite, wound and control treatments were administered to marked cells in each frame (Kuster et al., 2014), and the location of cells containing each treatment was recorded on the transparent plastic sheets. Capped cells were opened for treatment administration by cutting and lifting one side of the cell cap with a razor blade. Each cell received either one mite, one wound, or the control treatment. Mites introduced to cells were in the phoretic stage, and were collected from adult worker bees in non-experimental colonies by sugar shake (Dietemann et al., 2013; Fakhimzadeh, 2001). Mites were gently rinsed with a drop of clean water, and then introduced to cells using a fine-tipped paintbrush within approximately one hour of collection. Wounds that mimic *Varroa* mite feeding were administered within the brood cell using 50μm diameter capillary needles that mimic mite-inflicted feeding sites (Herrmann et al., 2005) and were administered on the dorsal side of the brood between the first abdominal segment and the second thoracic segment according to existing protocols (Dade, 2009). Control cells were opened just as mite and wound cells, but received neither mite nor wound treatment. All cells were sealed directly after treatment administration by pressing the cell cap against the cell wall with the edge of the razor blade.

Frames were returned to their hive of origin for 24 hours to allow bees to reseal the cell caps and were then transferred to an incubator maintained at 34ºC with 50% relative humidity (RH). Depending on availability, three to ten individuals from each brood type-by-treatment group were collected each day on days 4, 5, and 6 post-capping. These days were chosen because brood removal rates were highest on these three days in a preliminary behavioral study (data not shown). After careful removal of the cell cap and a portion of the cell wall, each individual brood was gently extracted from its cell using wide tipped, flexible forceps, and placed inside a 2mL screw top glass vial with silicone septa (Agilent). Extraction of brood cuticular chemicals was performed within one hour of brood collection, and no brood that was visibly damaged (e.g.: became discolored before or during chemical extraction) was used. Brood labeled as receiving the mite treatment was only collected if a live mite was found inside of the cell at the time of collection.

Cuticular Chemistry

Individual brood were submerged and soaked in hexane for 9 minutes to collect non-polar cuticular compounds. This soak time was established in preliminary experiments, as it provided sufficient time for collection of cuticular chemicals, but insufficient time for the appearance of additional chemical signals (thought to represent contamination from chemicals inside the brood). The volume of hexane used varied between approximately 0.5mL and 1.0 mL, depending on the volume needed to completely submerge each sample. After 9 minutes, the hexane extract was removed

from the brood and stored in a separate 2mL glass vial. Brood and hexane samples were stored at -80°C until analysis. For extract analysis, hexane was evaporated overnight under a Fisher Hamilton SAFEAIRE® hood. Samples were reconstituted with 100µL (2012 and 2013) or 50µL (2014) of heptane after complete evaporation, but within 18 hours of placement in the hood. Heptane used for reconstitution was spiked with butyl butyrate (1 µL butyl butyrate per 10mL heptane) as an internal standard. For reconstitution, heptane was added to each sample vial for 3 minutes, and the resulting sample was transferred into a 400µL glass flat bottom insert (Agilent) using a gas tight 100µL syringe (Hamilton). Glass inserts were used to facilitate operation of the Gas Chromotography Mass Spectrometry (GC-MS) autosampler. Use of heptane rather than hexane prevented evaporation of the sample during operation of the autosampler. Samples were analyzed by GC-MS to characterize qualitative and quantitative features of the chemical brood extracts. All samples were analyzed at the University of North Carolina at Greensboro on a Shimadzu GC-MS QP2010S (operating at 0.97kV and acquiring m/z values from 40 to 650). Source and interface temperatures were 230°C and 250°C respectively. A 30m ZebronZB-5MS column with 0.25-mm diameter, 0.5-µm stationary phase thickness was used with helium as the carrier gas (column head pressure 70.2 kPa, total flow rate of 18.1 ml/min, column flow rate of 1.05ml/min, linear velocity 37.8cm/sec, purge flow 0.5mL/min, split ratio -1.0). Column oven temperature was 80°C, injection temperature was 280°C and injection mode was splitless. After a 1 minute hold, the oven temperature rose from 80 to 165°C at 15°C/min, and then from 165 to 320°C at 10°C/min, with a final hold at 320°C for 10 minutes.

Qualitative and quantitative data were collected for individual honey bee brood samples using GC-MS. Since qualitative differences in chemical profiles of honey bee brood were not expected (Salvy et al., 2001), only the internal standard and cuticular chemicals (n=33) that were reproducibly quantifiable in each of 10 samples from 2012 were used for analysis. For qualitative analysis (peak identification) we used the mass spectral libraries NIST 2005 and WILEY 2007, including supplementary editions. GC-MS post-run analysis software calculated match percentage using an algorithm that compared spectra of the compounds of interest with ions from known library spectra. The length of saturated hydrocarbons was confirmed based on comparison with an external standard composed of Supelco n-hydrocarbon mix (even-numbered alkanes from C8 to C40, diluted 1000:1 with heptane) and spiked with pentadecane (C15). For quantitative analysis we calculated, for each brood, the proportion of each chemical relative to the total chemicals measured. To calculate this proportion we divided the area under each peak (individual peak area) by the sum of the area under all 34 peaks of interest (Σ (peak areas)), including the 33 cuticular chemicals of interest and the internal standard butyl butyrate. Arcsin transformation was performed for normalization of the proportion data and stabilization of variance (Sokal & Rohlf, 1995), as demonstrated in the equation below.

$$\textit{Relative Peak Quantity} = \sin^{-1}(\sqrt{(\textit{individual peak area}/\Sigma(\textit{peak areas}))} \times 100$$

Triplicate runs for 2 samples indicated that error due to auto-sampler injection was minimal (average standard error for peak area between triplicate samples was ± 0.002).

Therefore repeated injections were deemed unnecessary, and each sample for the main analysis was analyzed only one time.

Virus Quantification

After hexane extraction, the quantity of deformed wing virus (DWV) in each individual honey bee brood was analyzed. For each sample, RNA was extracted, cDNA was synthesized, and quantitative PCR (qPCR) was performed. While standard kits were not used in 2013, the Sensifast™ cDNA Synthesis Kit and Sensifast™ SYBR Hi-ROX Kit were used in 2014. For RNA extraction in both 2013 and 2014, brood were transferred from glass vials to 2mL Eppendorf® tubes and homogenized with 0.5mL TRIzol™ (Ambion by Life Technologies) using a plastic pestle. Samples were incubated at room temperature for 10 minutes. After incubation, another 0.5mL TRIzol™ was added to each sample, and then samples were vortexed for twenty seconds on the highest speed setting (#10) of a Fisher Scientific Mini Vortexer. Next, 0.2mL chloroform was added to each sample, and samples were vortexed again. Samples were incubated at room temperature for 3 minutes, and then centrifuged at 12,000 RCF for 15 minutes. The top layer of each sample was then pipetted into a 1.5mL tube containing 0.5 mL of isopropanol. Samples were mixed and placed on ice for 15 minutes, and then centrifuged again at 12,000 RCF for 10 minutes. The supernatant was discarded, and 1mL of 75% ethanol was added to each pellet, before centrifuging again at 7,500 RCF for 5 minutes. The supernatant was discarded again, and samples were allowed to air dry for 15 minutes.

Next, 0.1mL of molecular grade water (G Biosciences) was added to each sample to resuspend the RNA pellets. Samples were stored at -80°C until used for cDNA synthesis.

To determine sample concentration and purity of the RNA extract for cDNA synthesis, a 1µL RNA aliquot from each extract was analyzed using a Nanodrop ND-1000 Spectrophotometer. The amount of sample needed for 2,000ng of RNA was then calculated and pipetted into 1.5mL Eppendorf® tubes. Water was added such that each sample reached a total volume of 8µL, and then 2.2µL of DNAse solution (Invitrogen) containing 1µL of DNAse, 1µL of DNAse buffer, and 0.2 µL of RNAse Out was added to each sample. Samples were then heated to 37°C for one hour, and then to 75°C for 10 minutes. Next, for 2013 samples only, 1µL of a solution containing 0.02 µL dT, 0.5 µL random hexamer, 0.2 µL dNTP, and 0.298 µL H_2O was added to each sample. All samples were incubated at 65°C for 5 minutes and then chilled on ice for 10 minutes. Next, for 2013 samples, 10µL of a Master Mix containing 4µL of First Strand buffer, 2µL of DTT, 0.5µL of Super Scriptase II (Applied Biosystems), and 3.5µL of molecular grade water was added to each sample. For 2014 samples, 9.8µL of Master Mix (Sensifast™) containing 4µL 5x TransAmp Buffer, 1µL Reverse Transcriptase and 4.8µL of molecular grade water was added to each sample. All samples were incubated at 42°C for 50 minutes, and then 70°C for 15 minutes. Samples were stored at -20°C until used for RT-qPCR analysis.

RT-qPCR was performed to determine the quantity of DWV in each sample. For each 2013 sample, 1µL of cDNA, 10µL Power SYBR Green Mix (Applied Biosystems), 8µL of water, 0.5µL of DWV forward primer (sequence: 5'-

GAGATTGAAGCGCATGAACA-3'), and 0.5µL of DWV reverse primer (sequence: 5'-TGAATTCAGTGTCGCCCATA-3') were added to 0.1ml MicroAmp Fast Optical 96-Well Reaction Plate tubes. For each 2014 sample, 2µL of cDNA, 10µL of 2x Sensifast™ SYBR Hi-ROX Mix, 7.2µL of water, 0.4µL of forward primer, and 0.4µL of reverse primer were added to 0.1ml MicroAmp Fast Optical 96-Well Reaction Plate tubes. Liquid was centrifuged to the bottom and samples were run through 40 cycles on an Applied Biosystems StepOne Plus qPCR machine set to SYBR as the passive agent. Samples were analyzed for DWV as well as for the reference gene Actin (forward primer sequence: 5'-TTGTATGCCAACACTGTCCTTT-3'; reverse primer sequence: 5'-TGGCGCGATGATCTTAATTT-3'). Each transcript for each sample was run in triplicate.

Based on RT-qPCR results, DWV was classified as either "low" or "high" for each sample. Samples were categorized as "low" for DWV if the cycle threshold (C_T) was undetermined in all three replicates, or if only one replicate contained a determined C_T with a secondary peak at the correct melting temperature (Tm). Samples were categorized as "high" for DWV if at least one replicate contained a determined C_T with the primary peak at the correct Tm, or if two or more replicates contained determined C_T values with secondary peaks at the correct Tm. DWV was placed on a continuous scale by calculating an average Delta C_T for each sample. Delta C_T was calculated for each sample by taking the average of the Delta C_T across all three replicates. When no C_T value was determined, a C_T of 40 (the number of cycles used for RT-qPCR) was used.

Delta C_T was calculated using the following equation, such that the higher the Delta C_T value, the greater the amount of DWV in the sample:

$$Delta\ C_T = C_T(Actin) - C_T(DWV)$$

Statistical Analysis

A full factorial MANOVA was used to understand the overall effects of treatment (mite, wound, and control), brood type (CON, HYG, and VSH), and their interactions on chemical profiles of honey bee brood. MANOVAs were performed across all three years, and for each year separately. As a follow-up to MANOVAs, two-way ANOVAs were used to determine which specific chemicals were significantly affected by treatment, brood type, and their interactions. These ANOVAs were also performed across all three years, and for each year separately. For those chemicals significantly affected by treatment in the two-way ANOVA run over all three years, post-hoc tests were used to explore pairwise differences in chemical quantities between the three treatment groups (mite, wound, and control). Two-way ANOVAs were also used to evaluate the effect of treatment on the chemicals significantly affected by treatment for each day (brood age) separately, and each brood type separately. Post-hoc tests were used to explore pairwise differences in chemical quantities between the three treatment groups (mite, wound, and control) for analyses of separate days and separate brood types. Bonferroni correction was used for all pairwise comparisons between treatments to control the family-wise error rate (using a corrected significance threshold of $p \leq 0.0167$). The datasets used for all analyses listed above included samples collected in 2012, 2013, and 2014.

The virus data from 2012 brood was incomplete and virus titers were much higher than that of the following years, so we deemed 2012 values unreliable and did not include them in the analysis. Thus, all virus data and analyses refer to brood samples from 2013 and 2014 only. Two-way ANOVA were used to determine the effects of DWV load on the mean quantity of chemicals significantly affected by treatment in 2013, 2014, and for both years combined. Two-way ANOVAS were then used to analyze the effect of DWV on the mean quantity of chemicals significantly affected by treatment for each day (brood age) separately, and for each brood type separately. For the single chemical quantitatively affected by DWV load when brood types were analyzed separately, Pearson product-moment correlation coefficients were computed to assess the relationship between chemical and DWV quantity for each brood type. A significance level of 0.05 was used for statistical tests that did not require Bonferroni correction. All statistical analyses were performed using IBM SPSS Statistics, Version 22.

Experiment II: Association between Stressor-Induced Signals and Hygienic Behavior

Sample Collection

Based on the findings from two-way ANOVAs in Experiment I, we predicted that the cuticular chemical elevated in response to honey bee stressors would be highest in uncapped brood, and the cuticular chemical that decreased in response to honey bee stressors would be lowest in uncapped brood. To test these predictions, a study was conducted over the summers of 2014 and 2015 to analyze whether the quantity of these chemicals differed between uncapped mite-infested brood, capped mite-infested brood,

and capped non-infested brood. As described above, mite and control treatments were applied to recently capped cells. In 2014, treatments were applied to single frames from each of two VSH colonies. In 2015, treatments were applied to two frames from a single VSH colony. VSH colonies were used because of their chemical response to mite-infestation, as was determined in Experiment I (see results). The cells containing introduced mites were monitored every few hours for uncapping from day 4 to day 6 post-capping. Brood was collected from mite-infested cells that were found uncapped and apparently unharmed (ie: removal by nurse bees had not yet begun). Each time an uncapped, mite-infested brood was collected, two control brood were also collected – one from a capped mite-infested cell, and one from a capped control cell without a mite. All brood were carefully extracted using wide tipped, flexible forceps, and placed inside a 2mL screw top glass vial with silicone septa (Agilent) for transport to the lab and hexane extraction. Extraction of brood cuticular chemicals was performed within one hour of sample collection as described above, ensuring by visual inspection that no brood was damaged from the collection process.

Cuticular Chemistry

As described above, individual brood were submerged and soaked in hexane for 9 minutes to collect non-polar cuticular compounds. All methodology used for chemical extraction and analysis followed the protocol described above. However for each sample in this experiment, only the relative quantities of the two peaks that were found to differ

significantly with treatment in a two-way ANOVA (see Experiment I results) were evaluated.

Virus Quantification

As described above, individual brood was analyzed for DWV content. All methodology used for RNA extraction, cDNA synthesis, RT-qPCR, and RT-qPCR analysis was the same as that listed for 2014, above.

Statistical Analysis

Two-way ANOVA were used to determine whether the mean quantity of the chemicals significantly affected by treatment (see Experiment I results) differed by cell type. Bonferroni correction was used for pairwise comparisons between cells types (brood from uncapped mite-infested cells, capped mite-infested cells, and capped control cells) to control the family-wise error rate (using a corrected significance threshold of $p \leq 0.0167$). All statistical analyses were performed using IBM SPSS Statistics, Version 22.

Results

Experiment I: Effects of Treatment and Brood Type on Honey Bee Chemical Profiles

A total of 33 chemicals from the cuticles of honey bee brood were characterized and quantified in this study (Table 3). Of these 33 chemicals, 12 were alkanes, 7 were alkenes, 13 were methylalkanes, and 1 was unidentified. A full factorial MANOVA was run on data collected from 2012, 2013, and 2014 to understand the effects of treatment,

brood type, and their interaction on the quantities of the 33 honey bee cuticular chemicals. When the model included data from all three years, significant effects on brood cuticular profiles were identified for treatment ($F_{(66,744)}$=1.51, p=0.007) and brood type ($F_{(66,744)}$=5.25, p<0.001), but no significant effects were identified for the treatment-by-brood type interaction ($F_{(132,1483)}$=0.72, p=0.991). When separate models were run for each year, colony effects were significant for 2012 ($F_{(66,138)}$=8.67, p<0.001), 2013 ($F_{(33,98)}$=3.63, p<0.001) and 2014 ($F_{(66,254)}$=4.91, p<0.001). No significant treatment effects were identified when models were run separately for 2012 ($F_{(66,138)}$=1.29, p=0.105), 2013 ($F_{(66,194)}$=1.27, p=0.109), and 2014 ($F_{(66,254)}$=1.09, p=0.315). Similarly, no significant effects were identified for the treatment-by-brood type interaction when separate models were run for 2012 ($F_{(132,277)}$=0.83, p=0.885), 2013 ($F_{(66,194)}$=0.81, p=0.837) and 2014 ($F_{(132,508)}$=0.77, p=0.966). Effects reported are based on the Wilks' Lambda statistic.

Two-way ANOVAs run on data collected from 2012, 2013, and 2014 indicated that of the 33 chemicals analyzed, 3 were significant for treatment effects and 14 were significant for brood type effects (Table 3). The 3 chemicals significantly affected by treatment were represented by peak numbers 32, 15, and 14 (P32, P15 and P14, respectively). When separate models were run for each year, treatment effects on P32 were marginally significant for 2012 ($F_{(2,101)}$=2.98, p=0.055), significant for 2013 ($F_{(2,129)}$=7.08, p=0.001), but not significant for 2014 ($F_{(2, 159)}$=0.30, p=0.743). In contrast, treatment effects on P15 were not significant for 2012 ($F_{(2,101)}$=2.67, p=0.074), 2013 ($F_{(2,129)}$=1.85, p=0.161) or 2014 ($F_{(2,159)}$=0.69, p=0.502), and treatment effects on P14

were significant for 2012 ($F_{(2,101)}$=4.79, p=0.010), but not 2013 ($F_{(2,129)}$=0.55, p=0.578) or 2014 ($F_{(2,159)}$=2.49, p=0.086).

Our first prediction was that honey bee stressors affect chemical brood signals. We used post-hoc tests to explore pairwise differences in chemical quantities between the three treatment groups for peaks significantly affected by treatment when the model was run over all three years. Mean P32 quantity was significantly higher in mite-infested brood than in control ($F_{(1,273)}$=7.33, p=0.007) or wounded ($F_{(1,272)}$=11.15, p=0.001) brood (Figure **8**). There was no difference in the mean P32 quantity between wounded and control brood ($F_{(1,277)}$=0.49, p=0.486) (Figure **8**). After Bonferroni correction, there was no difference in the mean P15 quantity between mite-infested and control brood ($F_{(,1,273)}$=5.48, p=0.020), mite infested and wounded brood ($F_{(1,272}$<0.01, p=0.980) or wounded and control brood ($F_{(1,277)}$=4.72, p=0.031). Mean P14 quantity was significantly lower in mite-infested brood than in control brood ($F_{(1,273)}$=6.01, p=0.015). There was no difference in the mean P14 quantity between mite-infested and wounded brood ($F_{(1,272)}$=2.13 p=0.146) or wounded and control brood ($F_{(1,277)}$=0.64, p=0.426).

We used two-way ANOVAs to explore the effect of treatment on the quantities of chemicals of interest (P32 and P14) for each day (brood age), separately because the effect of treatment on any signal that triggers hygienic behavior must be relatively consistent across the ages in which hygienic behavior is observed. P15 was not explored further since treatment effects were not significant in any single year in the ANOVAs reported above. Mean P32 quantity was significantly affected by treatment on days 4 ($F_{(2,153)}$=4.02, p=0.020) and 6 ($F_{(2,117)}$=5.62, p=0.005) post-capping, but was not

significantly affected by treatment on day 5 post-capping ($F_{(2,135)}$=1.32, p=0.270). Pair wise comparisons indicated that P32 quantity was significantly higher in mite infested brood than wounded brood on days 4 ($F_{(1,101)}$=7.08, p=0.009) and 6 ($F_{(1,79)}$=7.63, p=0.007). Mean P14 quantity was significantly affected by treatment on day 6 ($F_{(2,117)}$=3.96, p=0.022), but was not significantly affected by treatment on days 4 ($F_{(2,153)}$=1.01, p=0.365) or 5 ($F_{(2,135)}$=0.57, p=0.565) post-capping. Pair wise comparisons indicated that P14 quantity was significantly lower in mite infested brood that control brood on day 6 ($F_{(1,76)}$=7.77, p=0.007). Comparisons not listed were not significant after Bonferroni correction.

Our second prediction was that the effects of honey bee stressors on chemical brood signals are specific to honey bee brood type. We used two-way ANOVAs to explore the effect of treatment on the quantities of chemicals of interested (P32 and P14) for each brood type, separately. P15 was not explored further since treatment effects were not significant in any single year in the ANOVAs reported above. Treatment had a significant effect on P32 quantity for VSH brood ($F_{(2,157)}$=5.41, p=0.005), but did not have a significant effect for HYG ($F_{(2,160)}$=1.86, p=0.159) or CON ($F_{(2,88)}$=0.52, p=0.595) brood. Pairwise comparisons indicated that P32 was significantly higher in mite-infested VSH brood than in control ($F_{(1,105)}$=8.95, p=0.012) or wounded ($F_{(1,102)}$=7.19, p=0.009) VSH brood (Figure **9**). No significant difference was found in P32 quantity between wounded and control ($F_{(1,107)}$=0.06, p=0.813) VSH brood (Figure **9**). In HYG brood, no significant difference was found in P32 quantity between mite-infested and control ($F_{(1,106)}$=1.78, p=0.185) mite-infested and wounded ($F_{(1,107)}$=3.08, p=0.082) or wounded

and control ($F_{(1,107)}$=0.25, p=0.617) (Figure **9**). Similarly for CON brood, no significant difference was found in P32 quantity between mite-infested and control ($F_{(1,58)}$=0.26, p=0.612) mite-infested and wounded ($F_{(1,59)}$=1.28, p=0.262) or wounded and control ($F_{(1,59)}$=0.22, p=0.644) (Figure **9**). Treatment had no significant effect on P14 quantity for VSH ($F_{(2,157)}$=0.78, p=0.460), HYG ($F_{(2,160)}$=0.86, p=0.427), or CON ($F_{(2,88)}$=2.17, p=0.120) brood. No significant differences were found in P14 quantity between mite-infested and control, mite-infested and wounded, or wounded and control brood for VSH, HYG or CON brood (data not shown.)

The effects of DWV on P32, P15 and P14 were also explored using two-way ANOVAs across both years, and for 2013 and 2014 separately. When analyzed across both years, the mean P32 quantity was significantly higher in brood with high DWV levels than in brood with low DWV levels ($F_{(1,302)}$=7.69, p=0.006; Figure **10**). The mean P15 quantity was significantly lower in brood with high DWV levels than in brood with low DWV levels ($F_{(1,302)}$=4.63, p=0.032). DWV levels had no significant effect on P14 quantity ($F_{(1,302)}$=0.06, p=0.804). When analyzed for each year separately, mean P32 quantity was significantly higher in brood with high DWV levels than in brood with low DWV levels in 2013 ($F_{(1,133)}$=5.75, p=0.018) and marginally higher in brood with high DWV levels in 2014 ($F_{(1,167)}$=3.86, p=0.051). Mean P15 quantity was significantly lower in brood with high DWV levels than in brood with low DWV levels in 2013 ($F_{(1,133)}$=4.36, p=0.039) but not in 2014 ($F_{(1,167)}$=0.28, p=0.601). Similarly, mean P14 quantity was significantly lower in brood with high DWV levels than in brood with low DWV levels in 2013 ($F_{(1,133)}$=4.14, p=0.044) but not in 2014 ($F_{(1,167)}$=1.89, p=0.171). Two-way

ANOVAS were then used to analyze the effect of DWV on mean P32, P15 and P14 quantities for each day (brood age), separately. P32, P15 and P14 quantities were not significantly affected by DWV when analyzed for each brood age separately (data not shown). Since visual inspection of the results suggested that the DWV effect may be influenced by brood type, the effect of DWV on P32 and P15 were also explored using separate ANOVAs for each brood type. Mean P32 quantity was significantly higher in brood with high DWV levels than in brood with low DWV levels for HYG ($F_{(1,124)}$=12.45, p=0.001) and CON ($F_{(1,52)}$=6.52, p=0.014) brood, but not for VSH ($F_{(1,122)}$=0.17, p=0.679) brood (Figure **11**). However mean P15 quantity was not significantly different for brood with high and low DWV levels for VSH ($F_{(1,122)}$=1.53, p=0.219), HYG ($F_{(1,124)}$=3.00, p=0.086), or CON ($F_{(1,52)}$=0.50, p=0.485) brood. Pearson product-moment correlation coefficients were computed to assess the relationships between P32 quantity and DWV delta CT for each brood type. These analyses were performed for virus data samples where DWV titers were classified as "high." A statistically significant positive correlation was found between P32 quantity and delta CT for HYG brood (r=0.554, n=30, p=0.002), but not for CON (r=-0.021, n=22, p=0.928) or VSH (r=-0.054, n=62, p=0.677) brood (Figure **12**).

Experiment II: Association between Stressor-Induced Signals and Hygienic Behavior

Our third prediction was that honey bee stressors that affect chemical brood signals are associated with hygienic behavior. We used two-way ANOVA to test whether the mean quantity of P32 and P14 differed by cell type (uncapped mite-infested,

capped mite-infested, capped non-infested). There was a significant effect of brood cell type on mean P32 quantity for both 2014 ($F_{(2,9)}$=5.668, p=0.026) and 2015 ($F_{(2,57)}$=13.755, p<0.001). Since results were the same for both years, the data was combined ($F_{(,2,69)}$=16.812, p<0.001). Uncapped mite-infested brood had a significantly higher mean relative percent P32 than did capped mite-infested ($F_{(1,46,)}$=16.956, p<0.001) or capped control ($F_{(1,46)}$=21.429, p<0.001) brood (Figure **13**). There was no significant difference between the mean relative percent P32 for capped mite-infested and capped control brood ($F_{(1,46)}$=0.654, p=0.423). There was no significant effect of cell type on mean quantity of P14 for 2014 ($F_{(2,9,)}$=1.424, p=0.290), 2015 ($F_{(2,57)}$=2.535, p=0.088), or when both years were combined ($F_{(2,69)}$=1.301, p=0.279). DWV was only detected in one capped mite-infested cell from 2014. In a subset of 17 brood tested from 2015 (n=6, 6 and 5 for uncapped mite-infested, capped mite-infested and non mite-infested controls, respectively), DWV was detected in 4 uncapped mite-infested cells, 4 capped mite-infested cells, and 0 capped control cells.

Discussion

In an effort to identify potential signals that trigger hygienic brood removal, we compared cuticular profiles for stressed and non-stressed individuals from three honey bee brood types. Specifically, we sought to determine whether honey bee stressors were associated with chemical brood signals, and whether any signals identified were specific to honey bee brood type. In an effort to determine whether stressor-induced signals were associated with hygienic behavior, we compared cuticular profiles of uncapped mite-

infested brood with those of capped mite-infested, and capped non-infested controls. Our hypothesis that hygienic behavior and related stressors are associated with chemical brood signals specific to brood type is supported by the elevation of P32 in the chemical profiles of mite-infested brood, brood with high DWV titers, and brood uncapped by nurse bees as well as by the differences in the stressor eliciting P32 between brood types. These results indicate that the increased hygienic removal apparent in hygienic honey bees may be driven by differences in the ability of brood within the hygienic colonies to signal stress via changes in production of cuticular chemicals.

Drastic quantitative changes in honey bee cuticular hydrocarbons occur as brood ages. However, for a chemical to be an effective trigger for hygienic behavior, it must have a relatively consistent response to stress across the ages in which hygienic behavior is observed. Of the three chemicals that varied quantitatively with treatment, P32 was the most robust over time, as treatment had a significant effect on P32 on days 4 and 6 post-capping. The robustness of this effect further supports our hypothesis that hygienic behavior and related stressors are associated with chemical brood signals, and supports the link between P32 and hygienic behavior, which is known to occur over many brood ages. The effects of treatment on P15 and P14 were less robust over time, as each was significantly affected by treatment on only one of the three days in which brood cuticles were analyzed. It is unclear why treatment did not affect P32 quantity on day 5 post-capping, though this and the significant effect of treatment on P15 for the same day may be related to the significant morphological changes (such as head formation) taking place at this time in honey bee brood development (Winston, 1991). In addition to the lack of

robust treatment effects for P15 and P14 over time, the effects of individual treatments on P15 mean quantity were not significant after Bonferroni correction, and neither DWV nor brood uncapping were associated with changes in P14 quantity. As a result we do not consider results regarding P15 and P14 to be sufficiently robust to add support our hypothesis that hygiene-related stressors are associated with chemical brood signals.

It has been postulated for over a decade that the trigger for hygienic behavior involves chemicals from the brood cuticle (Aumeier & Rosenkranz, 2001; Richard et al., 2008; Salvy et al., 2001; Schöning et al., 2012). For example, Aumeier et al. (2001) found no evidence that hygienic behavior resulted from odor or movement of *Varroa*, suggesting instead that the signal triggering hygienic behavior might originate from the brood. Richard et al. (2008) linked the injection of adult bees with lipopolysaccharide (bacterial coat protein) to variations in cuticular hydrocarbons and to increased aggression by nestmates. Schöning et al. (2012) found evidence that brood parasitized by highly virulent mites (mites with high DWV loads) were more likely to be removed than brood parasitized by less virulent mites, and linked parasitization by highly virulent mites with changes in chemical profiles of brood. Baracchi et al. (2012) found evidence of antiseptic removal of DWV-infected adult bees from the colony, and linked DWV infection to changes in cuticular hydrocarbons of adult bees, specifically an increase in high molecular weight molecules. Other studies have linked variation in brood chemicals to mite-infestation (Annoscia et al., 2012; Salvy et al., 2001) and DWV (Schöning et al., 2012). However, this is the first study to link a cuticular brood chemical to multiple common honey bee stressors, as well as the first study to successfully link a cuticular

brood chemical (notably the *same* chemical associated with honey bee stressors) to hygienic behavior.

Of the 32 chemicals extracted and identified in our study, 12 were alkanes, 7 were alkenes, and 13 were methylalkanes. All of these hydrocarbons have been previously identified as constituents of honey bee cuticles (Baracchi et al., 2012; Ferreira-Caliman et al., 2012; Piccolo et al., 2010; Richard et al., 2008; Salvy et al., 2001; Schmitt et al., 2007). P32 (tritricontene) is no exception, and has been found to differ quantitatively in the presence of stressors in multiple previous studies. Salvy et al (2001) found that honey bee larvae parasitized by more than one *Varroa* mite had higher relative proportions of P32 than non-parasitized larvae. Nazzi et al. (2002) reported a slightly higher proportion of P32 in honey bee larvae infested with *Varroa*, compared to non-infested controls. Richard et al. (2008) reported a significant increase in P32 on the cuticle of lipopolysaccharide-injected adults. While Baracchi et al. (2012) did not find evidence of a difference in P32 between DWV-infected and non-infected adult bees, they did link DWV infection to an increase in other high molecular weight molecules. The lack of evidence from other studies for differences in P32 quantity in response to stressors may be an effect of differences in chemical extraction methods, honey bee age (i.e.: analysis of adult honey bees), variability in P32 response between colonies, and/or colony types utilized in the study (McDonnell et al., 2013; Richard et al., 2012).

It is evident from our study that variation in P32 response between colony types occurs. In VSH bees P32 is elevated in response to mite treatment, while in HYG bees P32 is elevated in response to DWV. For VSH bees, which have been selected based on

suppression of mite reproduction, it is conceivable that an enhanced stress signal like P32 may have evolved in response to *Varroa* infestation. For example, colonies with brood capable of producing higher intensity signals in response to *Varroa* parasitization would be better able to interrupt the *Varroa* reproductive cycle through increased hygienic behavior, and thus would contain fewer mites and less reproductively-successful mites than non-VSH colonies. This is consistent with our finding that P32 is elevated in mite-infested VSH brood. It is less clear how an enhanced stress signal may have evolved in response to DWV in HYG bees, which have been selected based on freeze-killed brood assays, though a general response to damage may be involved. While reactions involved in hydrocarbon synthesis do happen on the order of minutes (Vaz et al., 1988), it is not feasible that large hydrocarbons are synthesized and transported to the cuticle in the instant of the freeze from the liquid nitrogen used in freeze-killed brood assays. It is possible that elevation of P32 on the cuticle of freeze-killed brood is a passive response that occurs after brood death. However, to our knowledge, P32 quantity has not been studied with respect to freeze-killed brood.

Pheromone volatility is important to many aspects of insect communication. For example, pheromones must be stable enough to convey information over time and space and to minimize sensory habituation, yet volatile enough to reach intended targets at a distance, and then be cleared from sensory structures such that sensory thresholds are restored (Gullan & Cranston, 2010; Howard & Blomquist, 1982; 2005; Wilson, 1965). The need for restoration of sensory thresholds after exposure to low-volatility compounds like P32 may explain the up-regulation of genes involved in metabolism in the antennae

of VSH nurse bees (Mondet et al., 2015). Pheromones with low volatility are generally characterized by longer chain-lengths, persist longer in the environment, and are often perceived through direct contact. In contrast, pheromones with high volatility are generally characterized by shorter chain-lengths, are more readily broken down, and are often perceived over longer distances (Blomquist & Vogt, 2003; Dani et al., 2001; Ginzel et al., 2003). Honey bee cell caps are composed of a porous mix of wax and silk (Jay, 1964; Langstroth, 1914). The porous nature of honey bee cell caps likely allows airflow between the inside and outside of the cell (Boecking et al., 1999; Langstroth, 1914), which may aid in communication between sealed brood and nurses. However nurse bees may also perceive low-volatility diseased brood signals like P32 through direct contact with the lipophilic hydrocarbon after its transmission from the brood cuticle to the wax cap of the brood cell. By pressing their thoraces against brood cell surfaces, nurse bees have been observed to increase the temperature of the wax cap by up to 3.2°C (Bujok et al., 2002). Increased movement of compounds towards nurse bees on the outside of the brood cell, be it through volatilization or movement of a solid through the wax, may be facilitated by the process of brood incubation, although this remains to be tested.

With the evidence for a link between hygiene-associated stressors and P32 provided here, there may be ways to artificially increase contact between important brood chemicals and patrolling nurse bees, and thus, there may be opportunities for improved control of honey bee parasites and diseases. For example, when applied at appropriate concentrations to honey bee cells, P32 could be used as a treatment for colonies with high mite loads, initiating uncapping and perhaps removal of brood in cells infested by mites.

A stimulus for uncapping may lead to greater removal of *Varroa* in honey bee colonies since uncapping behavior requires higher sensitivity to odors than removal behavior (Gramacho & Spivak, 2003), and since *Varroa* are likely easier to detect, by a greater number of nurses, in uncapped cells. Additionally, recapped cells have been associated with reduced *Varroa* reproductive success due to increased mortality of mite offspring (Harris et al., 2012), and possibly as a result of the movement of foundress *Varroa* between brood (which may prevent mating of *Varroa* offspring) before the cell is recapped (Kirrane et al., 2011).

Findings from our study may also lead to improved control of honey bee parasites and diseases through development of an improved selective breeding assay. Since removal rates are lower for mite-infested brood than for freeze-killed brood (Boecking & Drescher, 1992; Spivak & Downey, 1998), it follows that the olfactory signal triggering hygienic behavior is likely lower in live brood than in dead brood (Spötter et al., 2012). The non-specificity of current selection methods (Rinderer et al., 2010; Spötter et al., 2012) may be improved upon through the development of a new assay, which utilizes uncapping or removal of P32-treated cells as the primary selection criteria for hygienic colonies from which to breed. Theoretically, colonies that uncap more cells treated with low concentrations of P32 would have more bees with high sensitivity to diseased brood odor, and thus more bees capable of detecting and uncapping brood with low intensity stress signals, such as those originating from brood parasitized by mites and/or with covert DWV infections. A potential danger of this method would be selection for compulsive uncapping behavior that leads to removal of brood that are sufficiently

healthy to remain in the colony. However this may not be a substantial threat when selecting for a specific signal. Since workers that perform uncapping behavior have been shown to have greater olfactory sensitivity than workers that perform removal behavior (Gramacho & Spivak, 2003), it could be that despite high levels of uncapping, the less sensitive bees performing removal tasks would be left to make decisions regarding brood removal once a cell is uncapped and the severity of the problem inside is exposed. Since high levels of recapping are already seen in VSH bees (Villa et al., 2009) and recapping is associated with increased mite offspring mortality (Harris et al., 2012), compulsive uncapping followed by recapping of healthy individuals may actually be beneficial to colony health.

Development of techniques to enhance the control of honey bee parasites and diseases will improve honey bee health, increase colony population sizes, and facilitate overwintering success (Genersch et al., 2010; Harbo, 1986). Improved honey bee health and survival are beneficial environmentally, in terms of improved natural ecosystem maintenance and reduction of the use of harmful miticides in honey bee colonies, and economically, in terms of augmented crop pollination and increased honey bee product yield. In addition to providing a potential tool for enhanced control of honey bee parasites and diseases, this study improves our understanding of honey bee communication, and may provide useful insights regarding active compounds, olfactory sensitivity, and the fundamental mechanisms of intraspecific communication of other social insects.

References

Aizen MA, Garibaldi LA, Cunningham SA & Klein AM (2008) Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. Current Biology 18: 1572-1575.

Aizen MA & Harder LD (2009) The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Current Biology 19: 915-918.

Amdam GV, Hartfelder K, Norberg K, Hagen A & Omholt SW (2004) Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite *Varroa destructor* (Acari: Varroidae): a factor in colony loss during overwintering? Journal of Economic Entomology 97: 741-747.

Anderson D & Trueman J (2000) *Varroa jacobsoni* (Acari: Varroidae) is more than one species. Experimental and Applied Acarology 24: 165-189.

Annoscia D, Del Piccolo F & Nazzi F (2012) How does the mite *Varroa destructor* kill the honeybee *Apis mellifera*? Alteration of cuticular hydrcarbons and water loss in infested honeybees. Journal of Insect Physiology 58: 1548-1555.

Aumeier P & Rosenkranz P (2001) Scent or movement of *Varroa destructor* mites does not elicit hygienic behaviour by Africanized and Carniolan honey bees. Apidologie 32: 253-264.

Aumeier P, Rosenkranz P & Francke W (2002) Cuticular volatiles, attractivity of worker larvae and invasion of brood cells by *Varroa* mites. A comparison of Africanized and European honey bees. Chemoecology 12: 65-75.

Ayasse M, Engels W, Lübke G, Taghizadeh T & Francke W (1999) Mating expenditures reduced via female sex pheromone modulation in the primitively eusocial halictine bee, *Lasioglossum (Evylaeus) malachurum* (Hymenoptera: Halictidae). Behavioral Ecology and Sociobiology 45: 95-106.

Bailey L & Ball B (1991) Honey Bee Pathology. Academic Press, London.

Baracchi D, Fadda A & Turillazzi S (2012) Evidence for antiseptic behaviour towards sick adult bees in honey bee colonies. Journal of Insect Physiology 58: 1589-1596.

Biesmeijer J, Roberts S, Reemer M, Ohlemüller R, Edwards M, Peeters T, Schaffers A, Potts S, Kleukers R & Thomas C (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313: 351-354.

Blomquist GJ & Vogt RG (2003) Insect pheromone biochemistry and molecular biology: The biosynthesis and detection of pheromones and plant volatiles. Elsevier Academic Press, San Diego, CA.

Boecking O, Bienefeld K & Drescher W (2000) Heritability of the *Varroa*-specific hygienic behaviour in honey bees (Hymenoptera: Apidae). Journal of Animal Breeding and Genetics 117: 417-424.

Boecking O & Drescher W (1992) The removal response of *Apis mellifera* L. colonies to brood in wax and plastic cells after artificial and natural infestation with *Varroa jacobsoni* Oud. and to freeze-killed brood. Experimental & Applied Acarology 16: 321-329.

Boecking O & Genersch E (2008) Varroosis–the ongoing crisis in bee keeping. Journal für Verbraucherschutz und Lebensmittelsicherheit 3: 221-228.

Boecking O, Rosenkranz P & Sasaki M (1999) The pore in the hard conical *Apis cerana* drone capping results from a spinning process. Apidologie 30: 513-519.

Bowen-Walker P, Martin S & Gunn A (1999) The Transmission of Deformed Wing Virus between Honeybees (*Apis mellifera* L.) by the Ectoparasitic Mite *Varroa jacobsoni* Oud. Journal of Invertebrate Pathology 73: 101-106.

Bujok B, Kleinhenz M, Fuchs S & Tautz J (2002) Hot spots in the bee hive. Naturwissenschaften 89: 299-301.

Chen Y, Pettis JS, Evans JD, Kramer M & Feldlaufer MF (2004) Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. Apidologie 35: 441-448.

Collins AM, Pettis JS, Wilbanks R & Feldlaufer MF (2004) Performance of honey bee (*Apis mellifera*) queens reared in beeswax cells impregnated with coumaphos. Journal of Apicultural Research 43: 128-134.

Council NR (2006) Status of Pollinators in North America: National Academies Press, Committee on the Status of Pollinators in North America., USA.

Cremer S, Armitage SAO & Schmid-Hempel P (2007) Social Immunity. Current Biology 17: R693-R702.

D'Aubeterre JP, Myrold DD, Royce LA & Rossignol PA (1999) A scientific note of an application of isotope ratio mass spectrometry to feeding by the mite, *Varroa jacobsoni* Oudemans, on the honeybee, *Apis mellifera* L. Apidologie 30: 351-352.

Dade HA (2009) Anatomy and dissection of the honey bee (revised edition). International Bee Research Association, Cardiff, UK.

Dani FR, Jones GR, Destri S, Spencer SH & Turillazzi S (2001) Deciphering the recognition signature within the cuticular chemical profile of paper wasps. Animal Behaviour 62: 165-171.

De Jong D, De Jong P & Goncalves L (1982) Weight loss and other damage to developing worker honeybees from infestation with *Varroa jacobsoni*. Journal of Apicultural Research 21: 165-167.

De Miranda JR & Genersch E (2010) Deformed Wing Virus. Journal of Invertebrate Pathology 103: S48-S61.

Dietemann V, Nazzi F, Martin SJ, Anderson DL, Locke B, Delaplane KS, Wauquiez Q, Tannahill C, Frey E & Ziegelmann B (2013) Standard methods for *Varroa* research. Journal of Apicultural Research 52: 1.

Dietemann V, Pflugfelder J, Anderson D, Charrière J-D, Chejanovsky N, Dainat B, De Miranda JR, Delaplane K, Dillier F-X & Fuch S (2012) *Varroa destructor*: research avenues towards sustainable control. Journal of Apicultural Research 51: 125-132.

Fakhimzadeh K (2001) Effectiveness of confectioner sugar dusting to knock down *Varroa destructor* from adult honey bees in laboratory trials. Apidologie 32: 139-148.

Ferreira-Caliman M, Turatti I, Lopes N, Zucchi R & Nascimento F (2012) Analysis of insect cuticular compounds by non-lethal solid phase micro extraction with styrene-divinylbenzene copolymers. Journal of Chemical Ecology 38: 418-426.

Francis B, Blanton W, Littlefield J & Nunamaker R (1989) Hydrocarbons of the cuticle and hemolymph of the adult honey bee (Hymenoptera: Apidae). Annals of the Entomological Society of America 82: 486-494.

Francis B, Blanton W & Nunamaker R (1985) Extractable surface hydrocarbons of workers and drones of the genus Apis. Journal of Apicultural Research 24: 13-26.

Frey E, Odemer R, Blum T & Rosenkranz P (2013) Activation and interruption of the reproduction of *Varroa destructor* is triggered by host signals (*Apis mellifera*). Journal of Invertebrate Pathology.

Garedew A, Schmolz E & Lamprecht I (2004) The energy and nutritional demand of the parasitic life of the mite *Varroa destructor*. Apidologie 35: 419-430.

Genersch E, Von Der Ohe W, Kaatz H, Schroeder A, Otten C, Büchler R, Berg S, Ritter W, Mühlen W & Gisder S (2010) The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41: 332-352.

Gilliam M, Taber III S & Richardson GV (1983) Hygienic behavior of honey bees in relation to chalkbrood disease. Apidologie 14: 29-39.

Gilliam M, Taber S, Lorenz BJ & Prest DB (1988) Factors affecting development of chalkbrood disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*. Journal of Invertebrate Pathology 52: 314-325.

Ginzel MD, Blomquist GJ, Millar JG & Hanks LM (2003) Role of contact pheromones in mate recognition in *Xylotrechus colonus*. Journal of Chemical Ecology 29: 533-545.

Gisder S, Aumeier P & Genersch E (2009) Deformed wing virus: replication and viral load in mites (*Varroa destructor*). Journal of General Virology 90: 463-467.

Goode K, Huber Z, Mesce KA & Spivak M (2006) Hygienic behavior of the honey bee (*Apis mellifera*) is independent of sucrose responsiveness and foraging ontogeny. Hormones and Behavior 49: 391-397.

Gramacho KP & Spivak M (2003) Differences in olfactory sensitivity and behavioral responses among honey bees bred for hygienic behavior. Behavioral Ecology and Sociobiology 54: 472-479.

Grixti JC, Wong LT, Cameron SA & Favret C (2009) Decline of bumble bees (Bombus) in the North American Midwest. Biological Conservation 142: 75-84.

Gullan PJ & Cranston PS (2010) The Insects: An Outline of Entomology. 4th Edition edn. John Wiley & Sons, West Sussex, UK.

Haarmann T, Spivak M, Weaver D, Weaver B & Glenn T (2002) Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. Journal of Economic Entomology 95: 28-35.

Harbo JR (1986) Effect of population size on brood production, worker survival and honey gain in colonies of honeybees. Journal of Apicultural Research 25: 22-29.

Harbo JR & Harris JW (2001) Resistance to *Varroa destructor* (Mesostigmata: Varroidae) when mite-resistant queen honey bees (Hymenoptera: Apidae) were free-mated with unselected drones. Journal of Economic Entomology 94: 1319-1323.

Harbo JR & Harris JW (2009) Responses to *Varroa* by honey bees with different levels of Varroa Sensitive Hygiene. Journal of Apicultural Research 48: 156.

Harris JW (2007) Bees with *Varroa* Sensitive Hygiene preferentially remove mite infested pupae aged ≤ five days post capping. Journal of Apicultural Research 46: 134-139.

Harris JW (2008) Effect of brood type on *Varroa*-sensitive hygiene by worker honey bees (Hymenoptera: Apidae). Annals of the Entomological Society of America 101: 1137-1144.

Harris JW, Danka RG & Villa JD (2012) Changes in infestation, cell cap condition, and reproductive status of *Varroa destructor* (Mesostigmata: Varroidae) in brood exposed to honey bees with *Varroa* sensitive hygiene. Annals of the Entomological Society of America 105: 512-518.

Herrmann M, Kanbar G & Engels W (2005) Survival of honey bee (*Apis mellifera*) pupae after trypan blue staining of wounds caused by *Varroa destructor* mites or artificial perforation. Apidologie 36: 107-111.

Highfield AC, El Nagar A, Mackinder LC, Laure M-LN, Hall MJ, Martin SJ & Schroeder DC (2009) Deformed wing virus implicated in overwintering honeybee colony losses. Applied and Environmental Microbiology 75: 7212-7220.

Howard RW (1993) Cuticular hydrocarbons and chemical communication. University of Nebraska Press, Lincoln, NE.

Howard RW & Blomquist GJ (1982) Chemical ecology and biochemistry of insect hydrocarbons. Annual Review of Entomology 27: 149-172.

Howard RW & Blomquist GJ (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons Annual Review of Entomology 50: 371-393.

Ibrahim A, Reuter GS & Spivak M (2007) Field trial of honey bee colonies bred for mechanisms of resistance against *Varroa destructor*. Apidologie 38: 67-76.

Ifantidis MD (1988) Some aspects of the process of *Varroa jacobsoni* mite entrance into honey bee (*Apis mellifera*) brood cells. Apidologie 19: 387-396.

Jay S (1964) The Cocoon of the Honey Bee, *Apis mellifera* L. The Canadian Entomologist 96: 784-792.

Kanbar G & Engels W (2003) Ultrastructure and bacterial infection of wounds in honey bee (*Apis mellifera*) pupae punctured by *Varroa* mites. Parasitology Research 90: 349-354.

Kirrane MJ, De Guzman LI, Rinderer TE, Frake AM, Wagnitz J & Whelan PM (2011) Asynchronous development of honey bee host and *Varroa destructor* (Mesostigmata: Varroidae) influences reproductive potential of mites. Journal of Economic Entomology 104: 1146-1152.

Kuster RD, Boncristiani HF & Rueppell O (2014) Immunogene and viral transcript dynamics during parasitic *Varroa destructor* mite infection of developing honey bee (*Apis mellifera*) pupae. The Journal of Experimental Biology 217: 1710-1718.

Langstroth LL (1914) Langstroth on the Hive and the Honey-bee: A Bee-keeper's Manual. Hopkins, Bridgman & Company.

Le Conte Y, Huang Z, Roux M, Zeng Z, Christidès J-P & Bagnères A-G (2015) *Varroa destructor* changes its cuticular hydrocarbons to mimic new hosts. Biology Letters 11: 20150233.

LeConte Y & Hefetz A (2008) Primer pheromones in social hymenoptera. Annual Review of Entomology 53: 523-542.

Lee KV, Steinhauer N, Rennich K, Wilson ME, Tarpy DR, Caron DM, Rose R, Delaplane KS, Baylis K & Lengerich EJ (2015) A national survey of managed honey bee 2013–2014 annual colony losses in the USA. Apidologie 46: 292-305.

Lockey KH (1988) Lipids of the insect cuticle: origin, composition and function. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 89: 595-645.

Losey JE & Vaughan M (2006) The economic value of ecological services provided by insects. BioScience 56: 311-323.

Martin C, Salvy M, Provost E, Bagnères A-G, Roux M, Crauser D, Clement J-L & Le Conte Y (2001) Variations in chemical mimicry by the ectoparasitic mite *Varroa jacobsoni* according to the developmental stage of the host honey-bee *Apis mellifera*. Insect Biochemistry and Molecular Biology 31: 365-379.

Martin SJ (2001) The role of *Varroa* and viral pathogens in the collapse of honeybee colonies: a modelling approach. Journal of Applied Ecology 38: 1082-1093.

Martin SJ, Highfield AC, Brettell L, Villalobos EM, Budge GE, Powell M, Nikaido S & Schroeder DC (2012) Global Honey Bee Viral Landscape Altered by a Parasitic Mite. Science 336: 1304-1306.

McDonnell CM, Alaux C, Parrinello H, Desvignes J-P, Crauser D, Durbesson E, Beslay D & Le Conte Y (2013) Ecto-and endoparasite induce similar chemical and brain neurogenomic responses in the honey bee (*Apis mellifera*). BMC Ecology 13: 25.

Memmott J, Waser NM & Price MV (2004) Tolerance of pollination networks to species extinctions. Proceedings of the Royal Society of London. Series B: Biological Sciences 271: 2605-2611.

Möckel N, Gisder S & Genersch E (2011) Horizontal transmission of deformed wing virus: pathological consequences in adult bees (*Apis mellifera*) depend on the transmission route. Journal of General Virology 92: 370-377.

Mondet F, Alaux C, Severac D, Rohmer M, Mercer AR & Le Conte Y (2015) Antennae hold a key to *Varroa*-sensitive hygiene behaviour in honey bees. Scientific Reports 5: 1-12.

Morse RA & Calderone NW (2000) The value of honey bees as pollinators of US crops in 2000. Bee Culture 128: 1-15.

Nascimento D & Nascimento F (2012) Acceptance threshold hypothesis is supported by chemical similarity of cuticular hydrocarbons in a stingless bee, *Melipona asilvai*. Journal of Chemical Ecology 38: 1432-1440.

Nation J, Sanford M & Milne K (1992) Cuticular hydrocarbons from *Varroa jacobsoni*. Experimental & Applied Acarology 16: 331-344.

Nazzi F, Brown SP, Annoscia D, Del Piccolo F, Di Prisco G, Varricchio P, Della Vedova G, Cattonaro F, Caprio E & Pennacchio F (2012) Synergistic parasite-pathogen

interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathogens 8: e1002735.

Nazzi F, Milani N & Della Vedova G (2002) (Z)-8-Heptadecene from infested cells reduces the reproduction of *Varroa destructor* under laboratory conditions. Journal of Chemical Ecology 28: 2181-2190.

Pettis J, Shimanuki WWH & Teel P (1991) Fluvalinate treatment of queen and worker honey bees (*Apis mellifera L*) and effects on subsequent mortality, queen acceptance and supersedure. Apidologie 22: 1-7.

Pettis JS, Collins AM, Wilbanks R & Feldlaufer MF (2004) Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. Apidologie 35: 605-610.

Piccolo F, Nazzi F, Vedova GD & Milani N (2010) Selection of *Apis mellifera* workers by the parasitic mite *Varroa destructor* using host cuticular hydrocarbons. Parasitology 137: 967-973.

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O & Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. Trends in ecology & evolution 25: 345-353.

Principal de D'Aubeterre J, Myrold DD, Royce LA & Rossignol PA (1999) A scientific note of an application of isotope ratio mass spectrometry to feeding by the mite, *Varroa jacobsoni* Oudemans, on the honeybee, *Apis mellifera* L. Apidologie 30: 351-352.

Richard F, Aubert A & Grozinger C (2008) Modulation of social interactions by immune stimulation in honey bee, *Apis mellifera*, workers. BMC Biology 6: 50.

Richard F-J, Holt HL & Grozinger CM (2012) Effects of immunostimulation on social behavior, chemical communication and genome-wide gene expression in honey bee workers (*Apis mellifera*). BMC Genomics 13: 558.

Rinderer TE, Harris JW, Hunt GJ & De Guzman LI (2010) Breeding for resistance to *Varroa destructor* in North America. Apidologie 41: 409-424.

Romer F (1991) The oenocytes of insects: differentiation, changes during molting, and their possible involvement in the secretion of molting hormone. AP Gupta edn. Rutgers University Press, New Brunswick, NJ.

Rosenkranz P, Aumeier P & Ziegelmann B (2010) Biology and control of *Varroa destructor*. Journal of Invertebrate Pathology 103: S96-S119.

Rosenkranz P & Renz M (2003) *Varroa destructor* infestation of adult bees, worker brood and drone brood during the season and consequences for treatment concepts. Apidologie 34: 509-510.

Ryabov EV, Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D, Mead A, Burroughs N & Evans DJ (2014) A virulent strain of deformed wing virus (DWV) of honeybees (*Apis mellifera*) prevails after *Varroa destructor*-mediated, or in vitro, transmission. PLoS Pathogens 10: e1004230.

Salvy M, Martin C, Bagneres A, Provost E, Roux M, Le Conte Y & Clement J (2001) Modifications of the cuticular hydrocarbon profile of *Apis mellifera* worker bees in the presence of the ectoparasitic mite *Varroa jacobsoni* in brood cells. Parasitology 122: 145-159.

Sammataro D, Untalan P, Guerrero F & Finley J (2005) The resistance of *Varroa* mites (Acari: Varroidae) to acaricides and the presence of esterase. International Journal of Acarology 31: 67-74.

Schatton-Gadelmayer K & Engels W (1988) Blood proteins and body weight of newly-emerged worker honeybees with different levels of parasitization of brood mites. Entomolgia Generalis 14: 93-101.

Schmitt T, Herzner G, Weckerle B, Schreier P & Strohm E (2007) Volatiles of foraging honeybees *Apis mellifera* (Hymenoptera: Apidae) and their potential role as semiochemicals. Apidologie 38: 164-170.

Schöning C, Gisder S, Geiselhardt S, Kretschmann I, Bienefeld K, Hilker M & Genersch E (2012) Evidence for damage-dependent hygienic behaviour towards *Varroa destructor*-parasitised brood in the western honey bee, *Apis mellifera*. The Journal of Experimental Biology 215: 264-271.

Sokal RR & Rohlf FJ (1995) The principles and practice of statistics in biological research. WH Freeman and Co., New York.

Soroker V & Hefetz A (2000) Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger*. Journal of Insect Physiology 46: 1097-1102.

Spivak M (1996) Honey bee hygienic behavior and defense against *Varroa jacobsoni*. Apidologie 27: 245-260.

Spivak M & Downey DL (1998) Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). Journal of Economic Entomology 91: 64-70.

Spivak M, Masterman R, Ross R & Mesce KA (2003) Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. Journal of Neurobiology 55: 341-354.

Spivak M & Reuter GS (2001a) Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. Apidologie 32: 555-565.

Spivak M & Reuter GS (2001b) *Varroa destructor* infestation in untreated honey bee (Hymenoptera: Apidae) colonies selected for hygienic behavior. Journal of Economic Entomology 94: 326-331.

Spleen AM, Lengerich EJ, Rennich K, Caron D, Rose R, Pettis JS, Henson M, Wilkes JT, Wilson M & Stitzinger J (2013) A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. Journal of Apicultural Research 52: 44-53.

Spötter A, Gupta P, Nürnberg G, Reinsch N & Bienefeld K (2012) Development of a 44K SNP assay focussing on the analysis of a *Varroa*-specific defence behaviour in honey bees (*Apis mellifera* carnica). Molecular Ecology Resources 12: 323-332.

Steinhauer N, Rennich K, Lee KV, Pettis J, Tarpy DR, Rangel J, Caron D, Sagili R, Skinner JA, Wilson ME, Wilkes JT, Delaplane KS, Rose R & van Engelsdorp D (2015) Colony Loss 2014-2015: Preliminary Results, Vol. 2015.

Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS, Rose R, Skinner JA, Tarpy DR & Wilkes JT (2014) A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. Journal of Apicultural Research 53: 1-18.

Sylvester HA, Watts RP, De Guzman L, Stelzer JA & Rinderer TE (1999) *Varroa* in the mating yard: II. The effects of *Varroa* and fluvalinate on drone mating competitiveness. American Bee Journal.

Topolska G, Ball B & Allen M (1995) Identification of viruses in bees from two Warsaw apiaries. Medycyna Weterynaryjna (Poland) 51: 145-147.

Tsuruda JM, Harris JW, Bourgeois L, Danka RG & Hunt GJ (2012) High-resolution linkage analyses to identify genes that influence *Varroa* sensitive hygiene behavior in honey bees. PLoS ONE 7: e48276.

vanEngelsdorp D, Caron D, Hayes J, Underwood R, Henson M, Rennich K, Spleen A, Andree M, Snyder R & Lee K (2012) A national survey of managed honey bee

2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. Journal of Apicultural Research 51: 115-124.

vanEnglesdorp D, Hayes Jr J, Underwood RM & Pettis J (2008) A survey of honey bee colony losses in the US, Fall 2007 to Spring 2008. PLoS ONE 3: e4071.

Vaz AH, Blomquist GJ & Reitz RC (1988) Characterization of the fatty acyl elongation reactions involved in hydrocarbon biosynthesis in the housefly, *Musca domestica* L. Insect Biochemistry 18: 177-184.

Villa JD, Danka RG & Harris JW (2009) Simplified methods of evaluating colonies for levels of *Varroa* Sensitive Hygiene (VSH). Journal of Apicultural Research 48: 162-167.

Wenner A & Bushing W (1996) *Varroa* mite spread in the United States. Bee Culture 124: 341-343.

Williams I (1994) The dependence of crop production within the European Union on pollination by honey bees. Agricultural Zoology Reviews (United Kingdom) 6: 229-257.

Wilson EO (1965) Chemical communication in the social insects. Science 149: 1064-1071.

Wilson-Rich N, Spivak M, Fefferman NH & Starks PT (2009) Genetic, individual, and group facilitation of disease resistance in insect societies. Annual Review of Entomology 54: 405-423.

Winston ML (1991) The Biology of the Honey Bee. Harvard University Press, Cambridge, Massachusetts.

Yang X & Cox-Foster D (2007) Effects of parasitization by *Varroa destructor* on survivorship and physiological traits of *Apis mellifera* in correlation with viral incidence and microbial challenge. Parasitology 134: 405-412.

Yang X & Cox-Foster DL (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proceedings of the National Academy of Sciences of the United States of America 102: 7470-7475.

Tables

Table 3. Compounds on the Cuticle of Control and Mite-Infested Honey Bee Brood, Including Mean Relative Quantity (±SD) of each Compound for Control and Mite-Infested Brood. The significance of post-hoc tests between control and mite treatments for each compound is also provided. The superscripts 1 and 2 represent significant treatment and brood type effects, respectively.

Peak	Compounds	Control Brood	Mite-Infested Brood	p-value
P1[2]	unidentified	2.60 ± 1.74	2.79 ± 1.68	1.00
P2[2]	nonadecane	1.90 ± 1.10	1.67 ± 1.08	0.24
P3	heneicosane	3.56 ± 1.69	3.34 ± 1.28	0.68
P4	tricosane	8.97 ± 2.18	9.03 ± 2.34	1.00
P5[2]	9- + 11-methyl tricosane	7.22 ± 1.67	7.07 ± 1.55	1.00
P6[2]	4-methyl tetracosane	1.36 ± 1.11	1.31 ± 1.14	1.00
P7[2]	pentacosene	4.38 ± 1.63	4.40 ± 1.86	1.00
P8[2]	pentacosane	16.27 ± 3.29	16.14 ± 3.52	1.00
P9[2]	11- + 13-methyl pentacosane	15.31 ± 2.62	15.09 ± 2.87	1.00
P10[2]	hexacosane	5.82 ± 0.93	5.76 ± 0.97	1.00
P11	12- + 14-methyl hexacosane	5.94 ± 1.03	5.86 ± 1.04	1.00
P12	heptacosene	5.23 ± 1.05	4.99 ± 1.12	0.18
P13	heptacosane	42.58 ± 6.25	42.81 ± 6.70	1.00
P14[1,2]	11- + 13-Methyl heptacosane	37.03 ± 3.53	35.99 ± 3.73	0.07
P15[1]	5-methyl heptacosane	4.08 ± 0.66	3.88 ± 0.71	0.08
P16[2]	11,15-Dimethyl heptacosane	8.71 ± 1.20	8.42 ± 1.43	0.29
P17	7,x-dimethyl heptacosane	5.44 ± 1.09	5.23 ± 1.21	0.38
P18[2]	octacosane	5.33 ± 0.99	5.25 ± 1.24	1.00
P19[2]	12-methyloctacosane	11.36 ± 1.62	11.25 ± 2.22	1.00
P20	nonacosene	7.77 ± 1.60	7.40 ± 1.35	0.13
P21	nonacosane	31.25 ± 6.59	31.32 ± 7.34	1.00
P22	11- + 13- +15-methyl nonacosane	32.70 ± 5.99	32.13 ± 4.51	1.00
P23	11,17-dimethylnonacosane	3.53 ± 1.42	3.30 ± 1.43	0.55
P24	triacontene	4.22 ± 1.43	3.95 ± 1.55	0.40
P25	triacontane	6.99 ± 1.22	6.69 ± 1.49	0.18
P26	8-Heintriacontene	9.14 ± 2.07	9.14 ± 2.34	1.00
P27[2]	hentriacontane	11.49 ± 2.69	11.88 ± 3.14	0.81
P28	11- + 13- +15-Methylhentriacontane	21.70 ± 3.73	21.09 ± 3.65	0.55
P29	13,17-dimethyl hentriacontane	7.02 ± 2.92	6.58 ± 3.11	0.69
P30[2]	dotriacontene	4.49 ± 3.80	4.88 ± 4.44	1.00
P31	methyl dotriacontane	4.35 ± 2.34	4.03 ± 2.46	0.76
P32[1]	tritriacontene	7.21 ± 2.42	8.12 ± 3.23	0.02
P33	tritriacontane	13.00 ± 2.87	12.78 ± 3.00	1.00

Figures

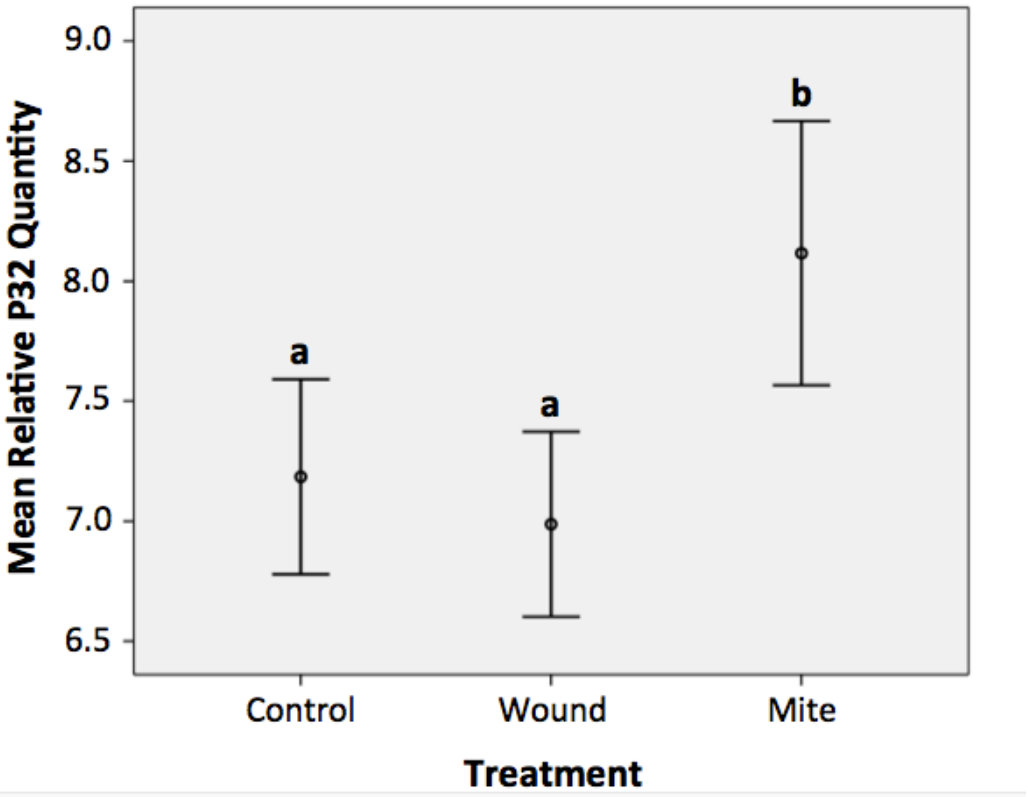

Figure 8. Effect of Treatment on Mean Relative P32 Quantity. For each mean, 95% CI intervals are provided. Different letters indicate significant differences from ANOVA after Bonferroni correction ($p<0.0167$). Sample size: Control n=140; Wounded n= 139; Mite n=135.

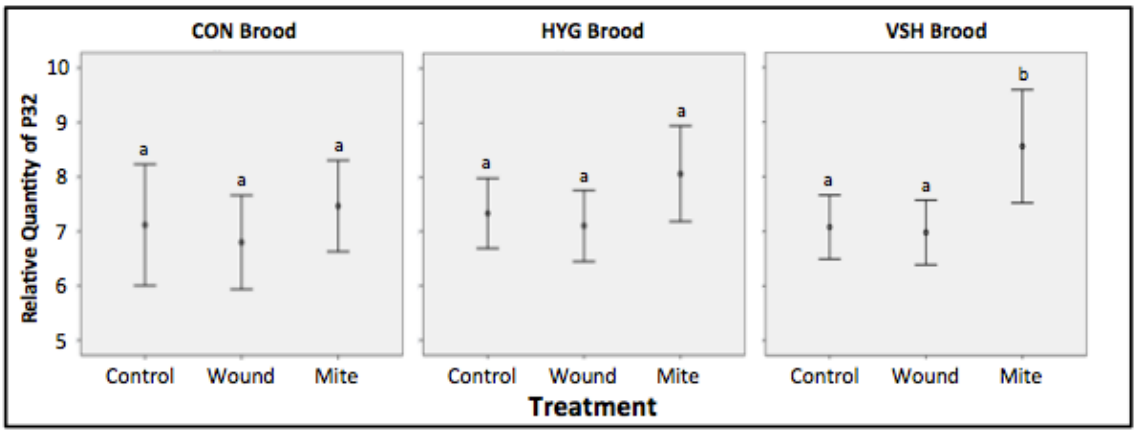

Figure 9. Mean P32 Quantity of Control and Mite-Infested Brood for CON, HYG and VSH Brood Types. For each mean, 95% CI intervals are provided. Different letters indicate a significant difference in mean P32 quantity between treatment groups within each brood type, from an ANOVA. Sample size: CON brood n=18 for both treatments; HYG brood n=41 for control and n=42 for mite-treated; VSH brood n=44 for control and n=39 for mite-treated.

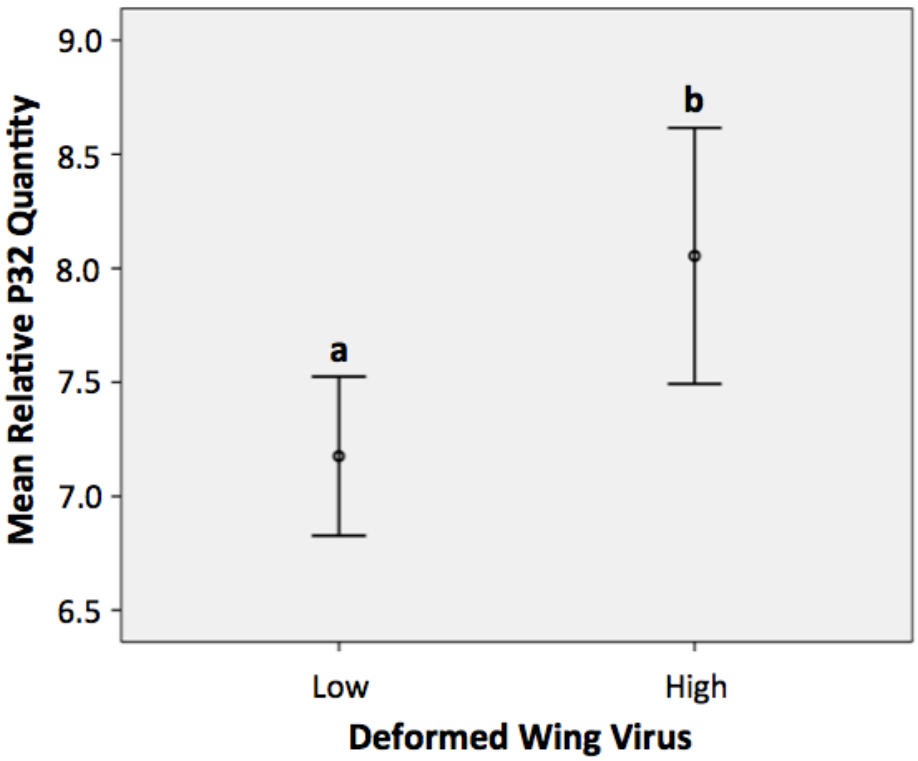

Figure 10. Effect of DWV Level on Mean Relative P32 Quantity. For each mean, 95% CI intervals are provided. Different letters indicate a significant difference from an ANOVA ($p<0.05$). Sample size: Low DWV n=190; High DWV n=114.

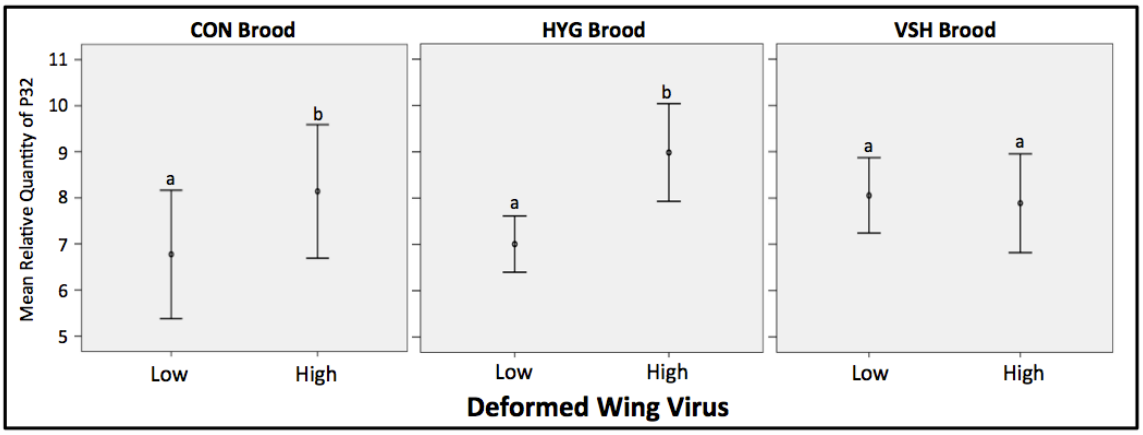

Figure 11. Mean Relative P32 Quantity of CON, HYG and VSH Brood with High and Low DWV Titers. For each mean, 95% CI intervals are provided. Different letters indicate a significant difference in mean P32 quantity between samples with high and low DWV titers. Sample size: CON brood n=32 for low DWV levels and n=22 for high DWV levels; HYG brood n=96 for low DWV levels and n=30 for high DWV levels; VSH brood n=62 for low DWV levels and n=62 for high DWV levels.

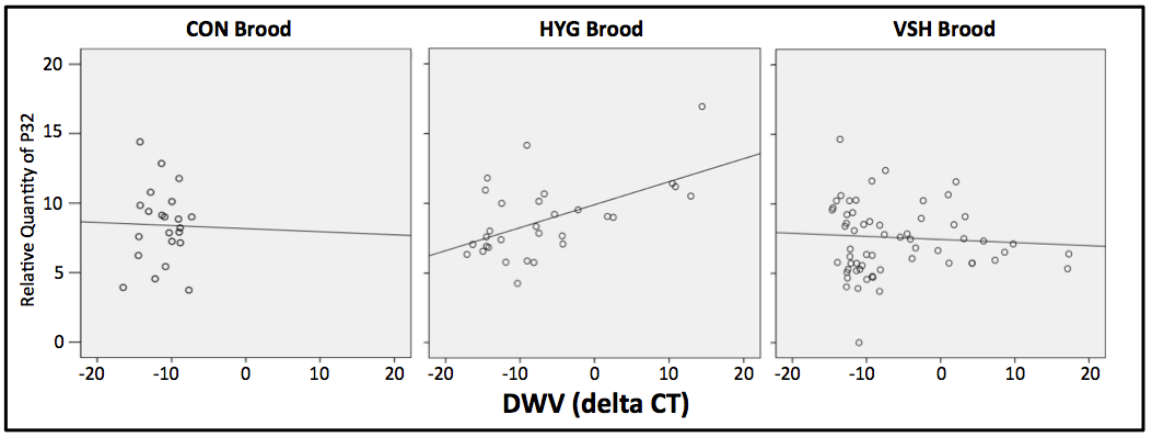

Figure 12. Correlation Between DWV Quantity and Relative P32 Quantity for CON, HYG and VSH Brood Types.

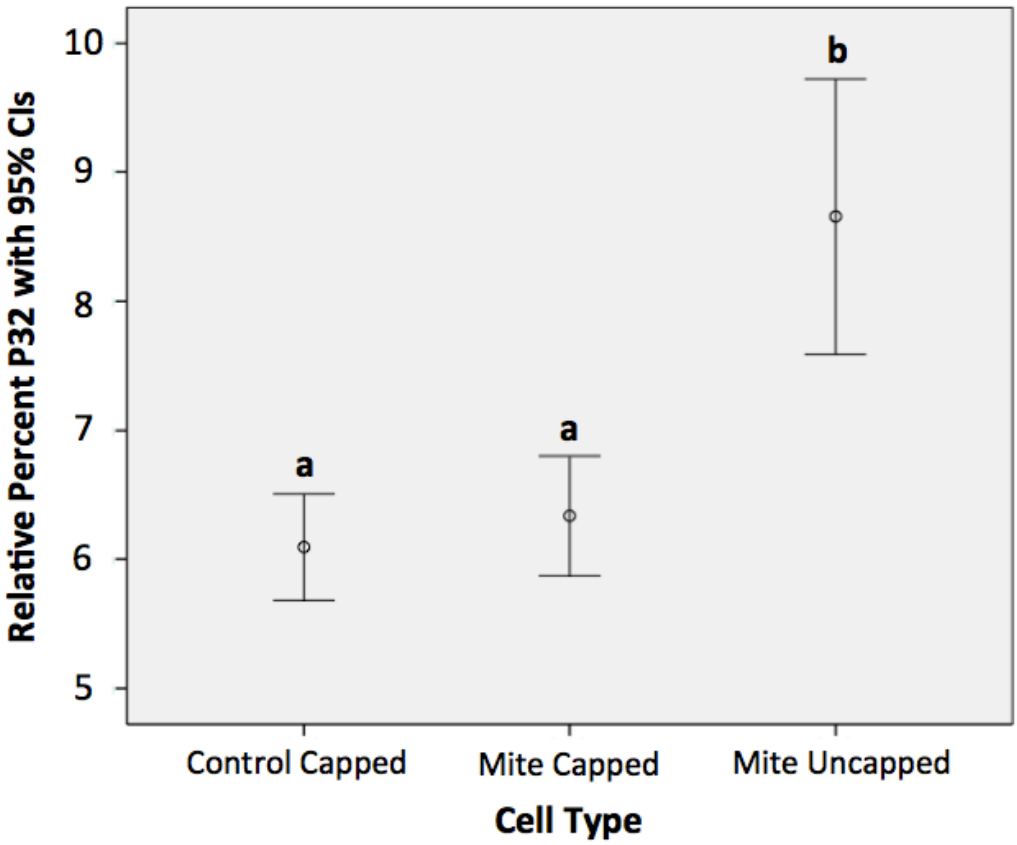

Figure 13. Relative P32 Quantity of the Brood from Control Capped, Mite-Infested Capped, and Mite-Infested Uncapped Cells. For each mean, 95% CI intervals are provided. Different letters indicate a significant difference ($p<0.0167$) in mean P32 quantity between cell types. Sample size: n=24 for each cell type.

CHAPTER IV

EFFECTS OF STEEL FOUNDATION WIRE ON ELEMENTAL CONTENT AND HYGIENIC REMOVAL OF HONEY BEE (*APIS MELLIFERA*) BROOD

This chapter has been submitted to the Journal Apidologie for publication, and is coauthored by Kaira M. Wagoner and Olav Rueppell.

Introduction

Recent honey bee (*Apis mellifera*) declines in the United States are primarily attributed to the individual and synergistic effects of several stressors including the introduction and spread of new parasites and associated pathogens, reduced genetic diversity, and environmental pressures such as agrochemical exposure and mismanagement of domestic colonies (Potts et al., 2010). Common beekeeper-induced stressors such as the use and accumulation of miticides (Burley et al., 2008; Collins et al., 2004; Haarmann et al., 2002; Mullin et al., 2010; Pettis et al., 2004) and the transportation and poor nutrition associated with commercial pollination routes (Foley et al., 2012; Rucker et al., 2011) are harmful to honey bee health. While it may not be practical to suspend the use of chemical miticides or restrict commercial pollination routes, certain beekeeping strategies can be used to improve honey bee health in the presence of stressors that are difficult to avoid (Delaplane et al., 2005; Di Pasquale et al., 2013; Schmidt et al., 1995). The scope of these strategies is as wide spread as the stressors themselves, ranging from breeding for hygienic behavior

(Spivak & Downey, 1998) and application of other Integrated Pest Management (IPM) strategies (Delaplane et al., 2005) to limiting and timing the use of certain agrochemicals thought to be harmful to honey bees (Hooven et al., 2013). These efforts share the common goal of increasing the number of healthy bees in each colony, as overwintering success is known to be positively correlated with colony population (Genersch et al., 2010; Harbo, 1986). However, despite these efforts, loss of honey bee colonies especially during overwintering continues to be high (Lee et al., 2015; Rucker et al., 2011; Spleen et al., 2013; Steinhauer et al., 2014).

Adult honey bees face many stressors outside of the nest such as predation (Visscher & Dukas, 1997), and exposure to pesticides (Johnson et al., 2010; Villa et al., 2000), insecticides (Rortais et al., 2005) and trace metals (van der Steen et al., 2012; 2015; Wang et al., 2013). Several trace metals have been shown to be toxic to adult honey bees, increasing mortality and oxidative stress, altering brain chemistry, and decreasing foraging and gustatory response (Hladun et al., 2013; Hladun et al., 2012; Nikolić et al., 2015; Søvik et al., 2015). Accumulation of trace metals in honey (Ioannidou et al., 2005; Islam et al., 2014; Özcan & Juhaimi, 2012) and otherwise within the hive is problematic because honey bees are highly susceptible to stress during development, when rapid growth and metabolism are taking place (Winston, 1991). Honey bee susceptibility to stress during development is evidenced by brood sensitivity to poor nutrition (Foley et al., 2012) and exogenous chemicals (Bromenshenk et al., 1991; Hladun et al., 2013; Thompson, 2003; Wu et al., 2011; Zhu et al., 2014). Many trace metals are thought to be acquired from food resources (Leita et al., 1996), such as

plants growing in contaminated soil (Hladun et al., 2012). However in some cases, metal contamination may be coming from within the hive itself (Özcan & Juhaimi, 2012). Based on preliminary observations, we hypothesized that the steel wires commonly used to add stability to wax foundation negatively affect honey bee brood health. More specifically, we predicted that brood in cells overlapping wires 1) incorporate metals from the wires into their tissue and 2) are removed at a higher rate than brood in cells not overlapping wires. To test our hypothesis we quantified the elemental content and measured the removal rates of brood developing in cells overlapping the foundation wires and in control cells directly adjacent to the cells that contained wire. Results from this study reveal a harmful practice common to modern beekeeping.

Materials and Methods

This experiment was conducted using four colonies in the summer of 2014. A repeat study in 2015 included three additional colonies. Each year experimental Langstroth colonies were newly established and equipped with 8 deep, wax-foundation frames (Brushy Mountain Bee Farm, Moravian Falls, NC). Each frame contained 9 vertical steel foundation wires. Each year, one colony was randomly selected for chemical analysis. From this colony, 3^{rd}-4^{th} larval instars (2014) or 4^{th}-5^{th} larval instars (2015) were collected: half from cells overlapping foundation wires and half from cells directly adjacent to foundation wires. In 2014, brood within each treatment was ordered visually from largest to smallest. To achieve the minimum weight of 0.1g required for chemical analysis, the brood were then pooled in pairs within each treatment, such that

the largest brood was paired with the smallest, the second largest was paired with the second smallest and so on. This pooling resulted in a total of 5 control and 5 wire samples, each containing two larvae. In 2015, brood within each treatment was randomly pooled in pairs, resulting in a total of 13 control and 13 wire samples, each containing two larvae. The ordered pooling methods from 2014 were not required in 2015 since the larvae were more developed, and thus easily reached the minimum weight requirement of 0.1g. Each year, a cell-length piece of wire void of wax was also collected for analysis. The brood and wire samples were sent to the United States Department of Agriculture (USDA) National Science Laboratory in Gastonia, NC for analysis using inductively coupled plasma mass spectrometry (ICP-MS). Samples were analyzed for arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), vanadium (V) and zinc (Zn). These elements were chosen based on previous research regarding trace elements leaching out of steel (Krachler & Shotyk, 2009). The elemental content of brood was compared between the two groups (wire and control) and the two years (2014 and 2015) using a one-way MANOVA. Following the MANOVA, individual one-way ANOVAs were used to compare between the two groups (wire and control) for each element, separately. One control brood sample (see sample #7 in Supplementary Table 1) was eliminated from the analyses as an outlier, as the amount of iron in the sample was over 5 times that of any other control sample from 2015. This sample also had the highest copper and manganese levels of all 2015 control samples, suggesting that the sample may have been contaminated.

From the center of each of the remaining colonies, a single frame containing 3rd-5th larval instars was collected. The total number of larvae present in cells overlapping and directly adjacent to steel foundation wires was determined for each frame. Frames were placed back into their respective colonies. After one week, frames were recollected and the number of brood remaining in the two treatment groups (overlapping and directly adjacent to each wire) was quantified. The removal of brood was compared between the two groups for each colony, and the difference was tested using Chi-square analysis. Chi-square analysis was then used to compare the removal of brood within each treatment group between colonies. Bonferroni correction was used for pairwise comparisons within each treatment group to control the family-wise error rate (using a corrected significance threshold of $p \leq 0.005$). Chi-square analysis was also used to compare removal of brood between the years for each treatment type separately. All statistical analyses were conducted using IBM SPSS Version 22.

Results

Brood discoloration and empty cells were commonly observed in the vicinity of the steel foundation wires present in wax foundation (Figure **14**). In 2014, the steel wire was found to contain at least trace amounts of all elements tested except cadmium, which, if present, was below the lower limit of detection. In 2015, the steel wire was found to contain at least trace amounts of all elements tested except for arsenic, cadmium, and cobalt, which, if present, were below the lower limit of detection. The most abundant element found in the steel wire in both 2014 and 2015 was iron, which was over 170

times more abundant than any other element tested. Due to significant differences between the two study years 2014 and 2015 ($F=105.9$; d.f.=8,24; $p<0.001$), chemical data were analyzed separately for the two years. Wire treatment significantly increased the presence of elements within the brood in 2014 ($F= 105.4$; d.f.=8,1; $p=0.038$) and 2015 ($F=2.8$; d.f.=7,17; $p=0.02$). There was suggestive evidence in 2014 ($F=2.3$; d.f.=1,8; $p=0.08$) and significant evidence in 2015 ($F=17.3$; d.f.=1,23; $p<0.001$) that brood collected from cells overlapping steel foundation wires contained more iron than brood collected from control cells (Figure **15**). There was no statistical evidence in either 2014 or 2015 that the other elements tested were more common in brood collected from cells overlapping steel wires (Table 3).

Between 2014 and 2015, a total of 1092 brood cells overlapping foundation wires and 1119 control brood cells adjacent to but not overlapping foundation wires were analyzed for removal. Brood overlapping foundation wire was removed at an average rate of 73.2%, with rates of 78.4, 93.4, 76.1, 59.1, and 70.0% for colonies 1, 2, 3, 4 and 5 respectively (Figure 16). In contrast, brood adjacent to foundation wire was removed at an average rate of 11.4%, with rates of 5.4, 6.6, 7.1, 6.5 and 30% for colonies 1, 2, 3, 4 and 5, respectively (Figure 16). Within 7 days of cell capping, brood overlapping the metal wires was on average removed at a rate of 6.4 times the rate of brood adjacent to the metal wires. Wire had a significant effect on brood removal in colonies 1 ($\chi^2=280$; df=1; $p<0.001$), 2 ($\chi^2=184$; df=1; $p<0.001$), 3 ($\chi^2=217$; df=1; $p<0.001$), 4 ($\chi^2=171$; df=1; $p<0.001$) and 5 ($\chi^2=76$; df=1; $p<0.001$). The removal of control brood was significantly higher in colony 5 than it was in colonies 1, 2, 3, and 4, which did not differ from one

another (Figure 16). The removal of wire-associated brood was not significantly different in colonies 1, 3 and 5 (Figure 16). However the removal of wire-associated brood was significantly higher in colony 2 than it was in any other colony, and was significantly lower in colony 4 than it was in any other colony except colony 5, which it did not differ from significantly (Figure 16). On average, control brood was removed significantly less in 2014 than in 2015 (χ^2=34.1; df=1; p<0.001), and wire-associated brood was removed significantly more in 2014 than in 2015 (χ^2=37.5; df=1; p<0.001; Figure 16). Wire aligned with the cell center overlapped approximately half as many cells as wire unaligned with the cell center. On each side of a deep frame there are approximately 3,350 cells. In a deep frame containing 9 wires, wires overlapped between 198 cells (all wires aligned with the cell center) and 396 cells (all wires unaligned with the cell center). Thus, between approximately 5.9% and 11.8% of brood in colonies containing a comparable quantity of steel foundation wires overlap the wires. Since removal of wire associated brood is 61.8% higher than removal of control brood, an excess of between 3.6% and 7.3% of all brood in a colony containing a comparable quantity of steel foundation wires may be removed because of their position along a wire.

Discussion

Results presented here suggest that the use of steel wire within beeswax foundation as a way to support the wax comb leads to a significant reduction in the survival of honey bee brood in cells that overlap the wire. Our hypothesis that steel wires negatively affect brood health is supported by the higher iron content and the higher

removal rates associated with brood in cells overlapping steel foundation wires. To our knowledge, this is the first study to quantify chemical contaminants and honey bee brood removal associated with steel foundation wires, and to assess the potential impact of these effects at the colony level.

Our findings regarding elevated iron content of wire-associated brood are consistent with the fact that steel is an alloy composed of primarily iron and carbon, which was confirmed by ICP-MS analysis indicating that iron was the most abundant element in the steel wire. These results are also supported by previous research, which reported an average of 145,230 ppb more iron in honey collected from cells overlapping steel wires compared to honey collected from cells not in contact with steel wires (Özcan & Juhaimi, 2012). This is comparable to the differences in iron content between control and wire-associated brood of 473,840 ppb and 159,103 ppb that we measured in 2014 and 2015, respectively. While the direction of the effect of steel wires on brood iron content was consistent between the two years of our study, higher concentrations of iron were measured in brood collected in 2014 than in brood collected in 2015. This is somewhat surprising considering that larvae collected in 2014 were younger, and thus had less time contact time with steel wires than larvae collected in 2015. Higher iron content in the brood samples from 2014 suggests that metal composition of the wires may have changed between years, and thus that it may be possible to improve the composition of metal wires to reduce their harmful impact on honey bees. The possibility that metal composition changed between years is also supported by the presence of arsenic and cobalt in 2014, both of which were absent in the wire sample from 2015.

While the iron concentrations we measured are below the LC_{50} values reported for *Drosophila* (Jimenez-Del-Rio et al., 2010), LC_{50} values for iron in honey bees have not been established. One study reported that iron-induced generation of reactive oxygen species in the brain impairs honey bee learning and memory (Farooqui, 2008), however the study involved direct injection of iron into honey bee antennal lobes, and thus is difficult to use for comparison. Due to limitations of our experimental design and the absence of LC_{50} values for iron in honey bees, it is unclear whether iron from the wires caused non-lethal damage or brood death. It is also unclear what the likelihood of brood death would have been had the brood not been removed. Discoloration observed in brood overlapping steel wires may be a sign of death or necrosis, but may also represent the mere incorporation of iron into the larval tissue. Regardless, it is evident that the incorporation of iron into brood tissue plays a role in triggering brood removal.

Previous studies have estimated survival of larvae and of sealed honey bee brood for colonies containing >9,000 adult bees, to be approximately 91.7% and 98.5%, respectively (Fukuda & Sakagami, 1968; Harbo, 1986; Martin, 2001). The implied removal rates from these studies are lower than the average removal rate of 11.4% for control brood from our study, which took place in similarly sized colonies. Additionally, removal rates presented in this study may be underestimated, as our results were based only on brood removal over the first 5-7 days post-capping. Given the full 13-day sealed period, it is likely that our removal rates would have been even higher than those reported here. High removal rates overall may be a result of increased levels of *Varroa* and related diseases since the estimates for brood survival cited above were established (Boecking &

Genersch, 2008; De Miranda & Genersch, 2010; Neumann & Carreck, 2010; Potts et al., 2010). However the high average removal of control brood over expected rates seems to be driven largely by the elevated removal of control brood in colony 5. Elevated removal of control brood in colony 5 may be the result of higher *Varroa* infestation, disease incidence, and/or hygienic level of that colony. Thus, the variation in removal of control brood could be either a year effect, or a colony effect. Differences in the removal rates of wire-associated brood among colonies do not seem to be dependent on the removal rates of control brood. While the removal rates of colonies 1-4 do not differ for control brood, removal rates were relatively high for wire-associated brood in colony 2, and relatively low for wire-associated brood in colony 4. This suggests a lack of synergism between the wire and other stressors, perhaps indicating that the trigger for hygienic removal of wire associated brood is strong, and does not require a high recognition threshold on the part of the nurse bee performing the removal task. Like the variation in removal of control brood, the variation in the removal of wire-associated brood could be either a year effect, driven by the different iron concentrations in brood from 2014 and 2015, or a colony effect, driven by other variations such as colony hygiene level.

Though the negative effect of wire on the presence of brood has been referenced previously, the effect was formerly described as a reluctance of the queen to lay eggs in wire-associated cells (Delaplane, 2006), rather than a removal effect. It is possible that the wire removal effect diminishes over time with continued use of the same foundation wire, as the previously reported reluctance of queens to lay eggs in wire-associated cells was described to diminish over time (Delaplane, 2006). Our study was conducted only

with newly drawn out frames, and thus we are unable to draw conclusions regarding the longevity of the effect. However the longevity of the effect may be less important in light of the recommendation that beekeepers replace their frames every year to reduce disease and pesticide accumulation in the hive (Evans & Spivak, 2010). Either way, our findings revise a previous misconception about reluctance of the queen to lay eggs in wire-associated cells. The importance of this revision should not be underestimated, as the difference between a reluctance to lay eggs and the removal of mature larvae represents a significant difference in energetic cost to the colony. Increased brood removal is associated with resource loss, because food resources are required to sustain the wire-associated brood before removal, as well as to provide the energy needed by nurses to carry out the removal tasks. In a simple thought experiment, we can consider how steel foundation wires might change the quality and quantity of resources within a colony. In addition to honey, other resources stored in wire-associated cells may become contaminated. Additionally, time is spent removing brood rather than performing other in-hive tasks, which may lead to a greater need for nurse bees and thus fewer foragers available to collect resources for the colony. As a result, the change in quality and quantity of resources in colonies exposed to steel foundation wires may lead to a decrease in the size of large colonies, and may limit the ability of small colonies to grow. However the effects of wire on resource quality and quantity remain to be tested, and at this point are no more than speculation.

We have provided evidence that the use of steel foundation wires affects brood survival and have discussed potential consequences of this effect on resource quality and

acquisition. However steel foundation wires may also affect adult honey bee populations, either through 1) compromised health in adult bees exposed to wires as brood, but not removed, or 2) ingestion of or direct contact with unhealthy brood by nurse bees during the process of hygienic removal (Cremer et al., 2007; Schmickl & Crailsheim, 2001). Iron-containing nanoparticles have been found in the abdomens of adult worker bees, but not in brood or drone (Wang et al., 2013). The nature and distribution of these particles suggests that they may be essential constituents in the navigational systems of adult worker bees (Wang et al., 2013). Consequently, exposure to iron during development or as a nurse bee might compromise health and navigational abilities later in life. These and other effects on adult population size may be critical to honey bee colony survival, as even seemingly small differences in adult survivorship can make significant differences in colony demographics. Honey bee population models suggest that there is a critical point at the equilibrium between brood production and forager mortality beyond which colony failure is accelerated (Khoury et al., 2011). Furthermore, these models suggest that colony collapse operates as a positive feedback loop, in which small effects that might initiate the process of population decline are self-accelerating (Khoury et al., 2011), and should be avoided. Thus, any contribution of steel foundation wires to the reduction of foragers or the inability to maintain brood production at a rate sufficient to replace forager mortality may lead to rapid population decline and eventual collapse of the colony.

Reduction in brood survival, resource quality, resource quantity, and adult survival contribute to the reduction of colony population size. Since colony population

size is positively correlated with overwintering success (Delaplane et al., 2013; Genersch et al., 2010; Harbo, 1986), it could be that use of steel foundation wires in honey bee hives contributes, either alone or in combination with other stressors, to overwintering losses, although this remains to be tested. While numerous chemical and biological honey bee stressors are difficult for beekeepers to control, there are some practices that beekeepers can avoid in order to improve honey bee health, including the use of steel wire-stabilized wax foundation. Several alternatives to steel wire foundation are available to beekeepers, including plastic foundation, wireless wax foundation (including use of wooden stabilizers), and the option of foundationless beekeeping. Our results reveal a harmful practice common to modern beekeeping. Reducing the use of harmful and unnecessary practices such as the use of steel wire-stabilized wax foundation may increase colony population sizes, and improve the health and overwintering success of managed honey bee colonies.

References

Boecking O & Genersch E (2008) Varroosis–the ongoing crisis in bee keeping. Journal für Verbraucherschutz und Lebensmittelsicherheit 3: 221-228.

Bromenshenk J, Gudatis J, Carlson S, Thomas J & Simmons M (1991) Population dynamics of honey bee nucleus colonies exposed to industrial pollutants. Apidologie 22: 359-369.

Burley LM, Fell RD & Saacke RG (2008) Survival of honey bee (Hymenoptera: Apidae) spermatozoa incubated at room temperature from drones exposed to miticides. Journal of Economic Entomology 101: 1081-1087.

Collins AM, Pettis JS, Wilbanks R & Feldlaufer MF (2004) Performance of honey bee (*Apis mellifera*) queens reared in beeswax cells impregnated with coumaphos. Journal of Apicultural Research 43: 128-134.

Cremer S, Armitage SAO & Schmid-Hempel P (2007) Social Immunity. Current Biology 17: R693-R702.

De Miranda JR & Genersch E (2010) Deformed Wing Virus. Journal of Invertebrate Pathology 103: S48-S61.

Delaplane KS (2006) Honey Bees and Beekeeping: A Year in the Life of an Apiary. Third Edition edn. The University of Georgia, Center for Continuing Education, Georgia, USA.

Delaplane KS, Berry JA, Skinner JA, Parkman JP & Hood WM (2005) Integrated pest management against *Varroa destructor* reduces colony mite levels and delays treatment threshold. Journal of Apicultural Research 44: 157-162.

Delaplane KS, van der Steen J & Guzman-Novoa E (2013) Standard methods for estimating strength parameters of *Apis mellifera* colonies. Journal of Apicultural Research 52: 1-12.

Di Pasquale G, Salignon M, Le Conte Y, Belzunces LP, Decourtye A, Kretzschmar A, Suchail S, Brunet J-L & Alaux C (2013) Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? PLoS ONE 8: e72016.

Evans JD & Spivak M (2010) Socialized medicine: individual and communal disease barriers in honey bees. Journal of Invertebrate Pathology 103: S62-S72.

Farooqui T (2008) Iron-induced oxidative stress modulates olfactory learning and memory in honeybees. Behavioral Neuroscience 122: 433.

Foley K, Fazio G, Jensen AB & Hughes WO (2012) Nutritional limitation and resistance to opportunistic *Aspergillus* parasites in honey bee larvae. Journal of Invertebrate Pathology 111: 68-73.

Fukuda H & Sakagami SF (1968) Worker brood survival in honeybees. Researches on Population Ecology 10: 31-39.

Genersch E, Von Der Ohe W, Kaatz H, Schroeder A, Otten C, Büchler R, Berg S, Ritter W, Mühlen W & Gisder S (2010) The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41: 332-352.

Haarmann T, Spivak M, Weaver D, Weaver B & Glenn T (2002) Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. Journal of Economic Entomology 95: 28-35.

Harbo JR (1986) Effect of population size on brood production, worker survival and honey gain in colonies of honeybees. Journal of Apicultural Research 25: 22-29.

Hladun KR, Kaftanoglu O, Parker DR, Tran KD & Trumble JT (2013) Effects of selenium on development, survival, and accumulation in the honeybee (*Apis mellifera* L.). Environmental Toxicology and Chemistry 32: 2584-2592.

Hladun KR, Smith BH, Mustard JA, Morton RR & Trumble JT (2012) Selenium toxicity to honey bee (*Apis mellifera* L.) pollinators: effects on behaviors and survival. PLoS ONE 7: 1-10.

Hooven L, Sagili R & Johansen E (2013) How to Reduce Bee Poisoning from Pesticides: Oregon State University Extension Service.

Ioannidou M, Zachariadis G, Anthemidis A & Stratis J (2005) Direct determination of toxic trace metals in honey and sugars using inductively coupled plasma atomic emission spectrometry. Talanta 65: 92-97.

Islam M, Khalil M & Gan SH (2014) Toxic compounds in honey. Journal of Applied Toxicology 34: 733-742.

Jimenez-Del-Rio M, Guzman-Martinez C & Velez-Pardo C (2010) The effects of polyphenols on survival and locomotor activity in *Drosophila melanogaster* exposed to iron and paraquat. Neurochemical Research 35: 227-238.

Johnson RM, Ellis MD, Mullin CA & Frazier M (2010) Pesticides and honey bee toxicity–USA. Apidologie 41: 312-331.

Khoury DS, Myerscough MR & Barron AB (2011) A quantitative model of honey bee colony population dynamics. PLoS ONE 6: e18491.

Krachler M & Shotyk W (2009) Trace and ultratrace metals in bottled waters: survey of sources worldwide and comparison with refillable metal bottles. Science of the Total Environment 407: 1089-1096.

Lee KV, Steinhauer N, Rennich K, Wilson ME, Tarpy DR, Caron DM, Rose R, Delaplane KS, Baylis K & Lengerich EJ (2015) A national survey of managed honey bee 2013–2014 annual colony losses in the USA. Apidologie 46: 292-305.

Leita L, Muhlbachova G, Cesco S, Barbattini R & Mondini C (1996) Investigation of the use of honey bees and honey bee products to assess heavy metals contamination. Environmental Monitoring and Assessment 43: 1-9.

Martin SJ (2001) The role of *Varroa* and viral pathogens in the collapse of honeybee colonies: a modelling approach. Journal of Applied Ecology 38: 1082-1093.

Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R & Pettis JS (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS ONE 5: e9754.

Neumann P & Carreck NL (2010) Honey bee colony losses. Journal of Apicultural Research 49: 1-6.

Nikolić TV, Purać J, Orčić S, Kojić D, Vujanović D, Stanimirović Z, Gržetić I, Ilijević K, Šikoparija B & Blagojević DP (2015) Environmental effects on superoxide dismutase and catalase activity and expression in honey bee. Archives of Insect Biochemistry and Physiology 0: 1-14.

Özcan MM & Juhaimi FYA (2012) Determination of heavy metals in bee honey with connected and not connected metal wires using inductively coupled plasma atomic emission spectrometry (ICP–AES). Environmental Monitoring and Assessment 184: 2373-2375.

Pettis JS, Collins AM, Wilbanks R & Feldlaufer MF (2004) Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. Apidologie 35: 605-610.

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O & Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. Trends in Ecology & Evolution 25: 345-353.

Rortais A, Arnold G, Halm M-P & Touffet-Briens F (2005) Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. Apidologie 36: 71-83.

Rucker R, Thurman W & Burgett M (2011) Colony collapse and the economic implications of bee disease.

Schmickl T & Crailsheim K (2001) Cannibalism and early capping: strategy of honeybee colonies in times of experimental pollen shortages. Journal of Comparative Physiology A 187: 541-547.

Schmidt LS, Schmidt JO, Rao H, Wang W & Xu L (1995) Feeding preference and survival of young worker honey bees (Hymenoptera: Apidae) fed rape, sesame, and sunflower pollen. Journal of Economic Entomology 88: 1591-1595.

Søvik E, Perry CJ, LaMora A, Barron AB & Ben-Shahar Y (2015) Negative impact of manganese on honeybee foraging. Biology Letters 11: 20140989.

Spivak M & Downey DL (1998) Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). Journal of Economic Entomology 91: 64-70.

Spleen AM, Lengerich EJ, Rennich K, Caron D, Rose R, Pettis JS, Henson M, Wilkes JT, Wilson M & Stitzinger J (2013) A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. Journal of Apicultural Research 52: 44-53.

Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS, Rose R, Skinner JA, Tarpy DR & Wilkes JT (2014) A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. Journal of Apicultural Research 53: 1-18.

Thompson HM (2003) Behavioural effects of pesticides in bees–their potential for use in risk assessment. Ecotoxicology 12: 317-330.

Van der Steen JJ, de Kraker J & Grotenhuis T (2012) Spatial and temporal variation of metal concentrations in adult honeybees (*Apis mellifera* L.). Environmental Monitoring and Assessment 184: 4119-4126.

Van der Steen JJ, de Kraker J & Grotenhuis T (2015) Assessment of the Potential of Honeybees (*Apis mellifera* L.) in Biomonitoring of Air Pollution by Cadmium, Lead and Vanadium. Journal of Environmental Protection 6: 96.

Villa S, Vighi M, Finizio A & Serini GB (2000) Risk assessment for honeybees from pesticide-exposed pollen. Ecotoxicology 9: 287-297.

Visscher P & Dukas R (1997) Survivorship of foraging honey bees. Insectes Sociaux 44: 1-5.

Wang T-H, Jian C-H, Hsieh Y-K, Wang F-N & Wang C-F (2013) Spatial distributions of inorganic elements in honeybees (*Apis mellifera* L.) and possible relationships to dietary habits and surrounding environmental pollutants. Journal of Agricultural and Food Chemistry 61: 5009-5015.

Winston ML (1991) The Biology of the Honey Bee. Harvard University Press, Cambridge, Massachusetts.

Wu JY, Anelli CM & Sheppard WS (2011) Sub-lethal effects of pesticide residues in brood comb on worker honey bee (*Apis mellifera*) development and longevity. PLoS ONE 6: e14720.

Zhu W, Schmehl DR, Mullin CA & Frazier JL (2014) Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. PLoS ONE 9: e77547.

Tables

Table 4. Average Elemental Content of Control and Wire-Associated Brood from 2014 and 2015 (with Standard Deviations and Comparisons from Individual, One-Sided ANOVA).

Element	2014			2015		
	Control (ppb)	Wire (ppb)	ANOVA	Control (ppb)	Wire (ppb)	ANOVA
Arsenic (As)	82 ± 19	78 ± 40	$F_{(1,8)}$=0.04, p=0.42	17 ± 2	18 ± 3	$F_{(1,23)}$=0.02, p=0.45
Cadmium (Cd)	183 ± 218	78 ± 40	$F_{(1,8)}$=1.11, p=0.16	17 ± 2	18 ± 3	$F_{(1,23)}$=0.02, p=0.45
Chromium (Cr)	2,240 ± 2,913	3,995 ± 7,410	$F_{(1,8)}$=0.24, p=0.32	107 ± 164	43 ± 90	$F_{(1,23)}$=1.50, p=0.12
Cobalt (Co)	140 ± 123	78 ± 40	$F_{(1,8)}$=1.14, p=0.17	17 ± 2	18 ± 3	$F_{(1,23)}$=0.02, p=0.45
Copper (Cu)	11,318 ± 4,625	11,496 ± 8,377	$F_{(1,8)}$=0.00, p=0.48	6,938 ± 1,856	7,445 ± 1,196	$F_{(1,23)}$=0.65, p=0.21
Iron (Fe)	315,360 ± 258,895	789,200 ± 644,568	$F_{(1,8)}$=2.33, p=0.08	59,158 ± 61,008	218,262 ± 118,831	$F_{(1,23)}$=17.27, p<0.01
Lead (Pb)	1,918 ± 623	1,612 ± 1,251	$F_{(1,8)}$=0.24, p=0.32	100 ± 152	104 ± 97	$F_{(1,23)}$=0.01, p=0.47
Manganese (Mn)	4,704 ± 760	7,430 ± 5,106	$F_{(1,8)}$=1.39, p=0.14	8,620 ± 10,802	9,856 ± 9,199	$F_{(1,23)}$=0.10, p=0.38
Vanadium (V)	82 ± 19	78 ± 40	$F_{(1,8)}$=0.04, p=0.42	17 ± 2	18 ± 3	$F_{(1,23)}$=0.02, p=0.45
Zinc (Zn)	51,880 ± 7,195	60,900 ± 57,946	$F_{(1,8)}$=0.12, p=0.37	863,842 ± 1,307,772	721,477 ± 1,211,948	$F_{(1,23)}$=0.07, p=0.40

Figures

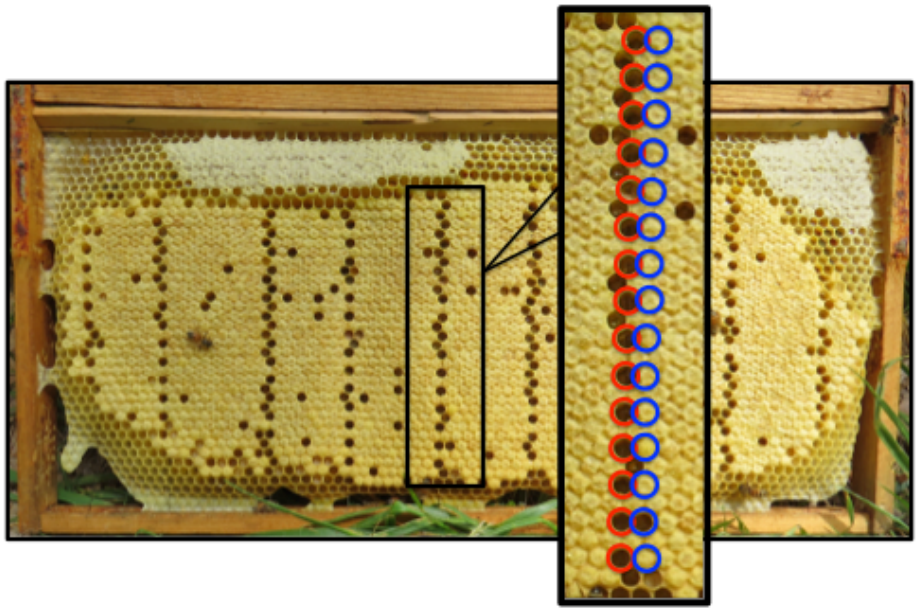

Figure 14. Effect of Foundation Wire on Brood Removal and Experimental Design to Select Contaminated and Control Cells. Red indicates brood cells overlapping the wire and blue indicates control brood cells adjacent to the wire.

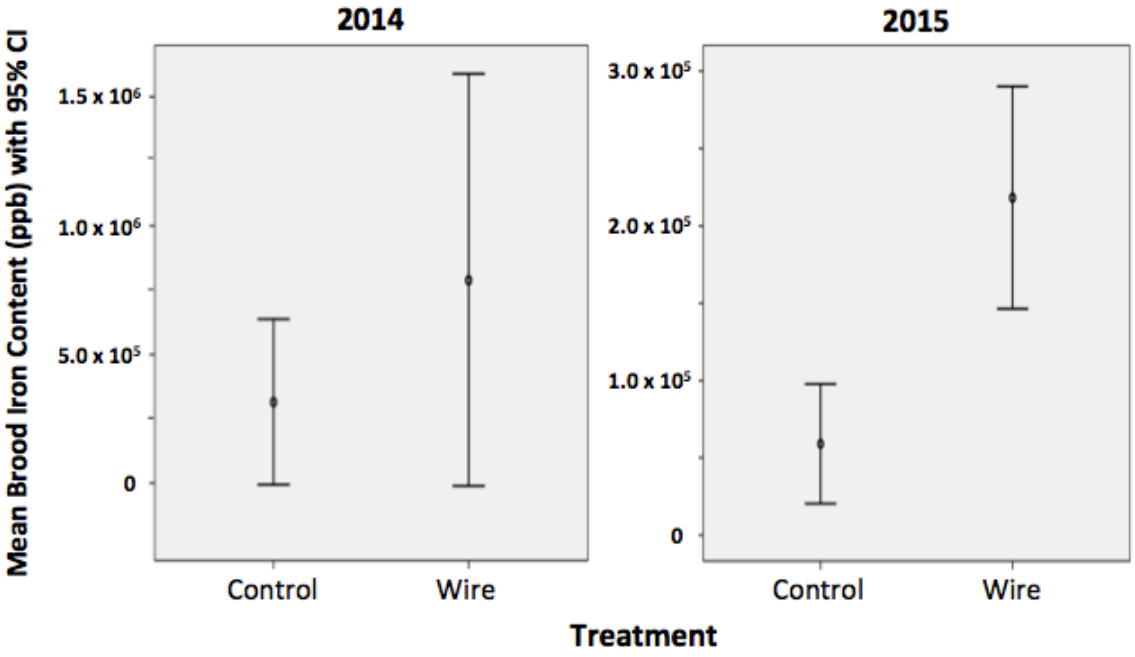

Figure 15. Mean Iron Content in Brood Collected from Cells Overlapping and Adjacent to Wires for 2014 and 2015, with 95% Confidence Intervals. Wire-associated brood has higher iron content than control brood, but some variability in the scale of the effect exists between years.

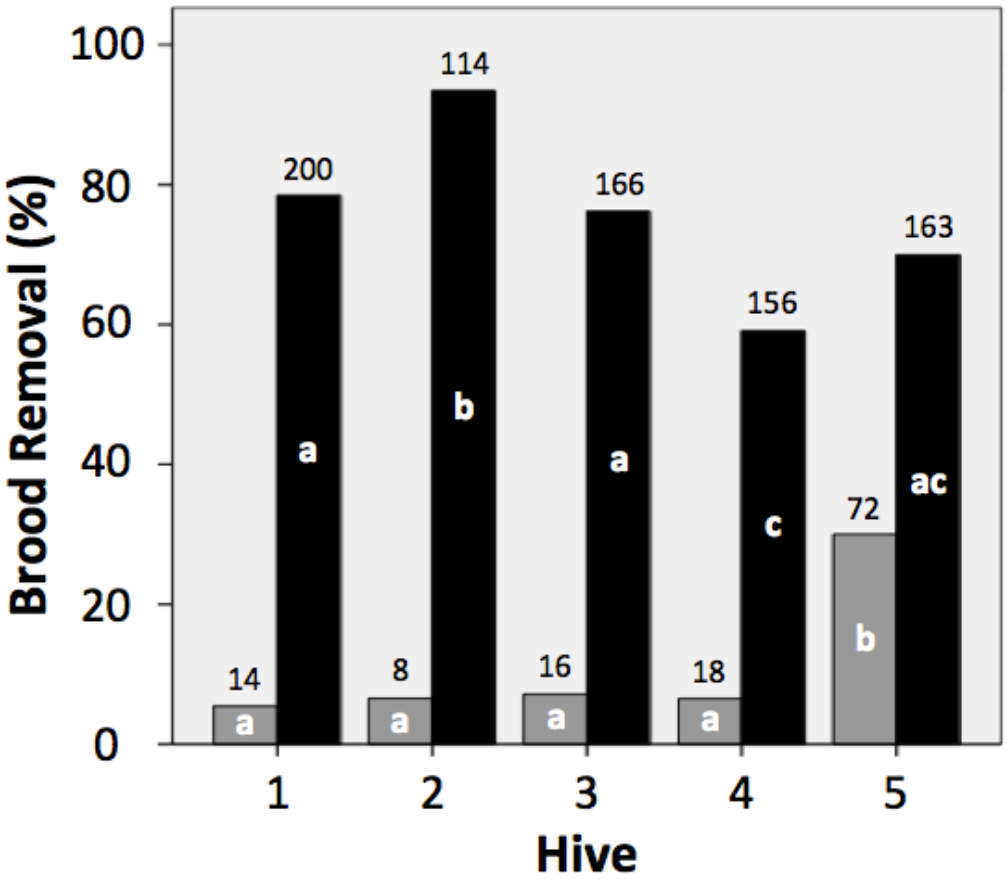

Figure 16. Percent Brood Removal Rate from Cells Overlapping (Black) and Adjacent to (Gray) Foundation Wire for Five Colonies. Brood in cells overlapping wire undergoes higher removal than brood in control cells, but some variability between colonies exists. Numbers above bars represent the absolute number of brood removed. Different letters indicate significant differences between colonies within each treatment group from Chi-square analysis after Bonferroni correction ($p<0.005$).

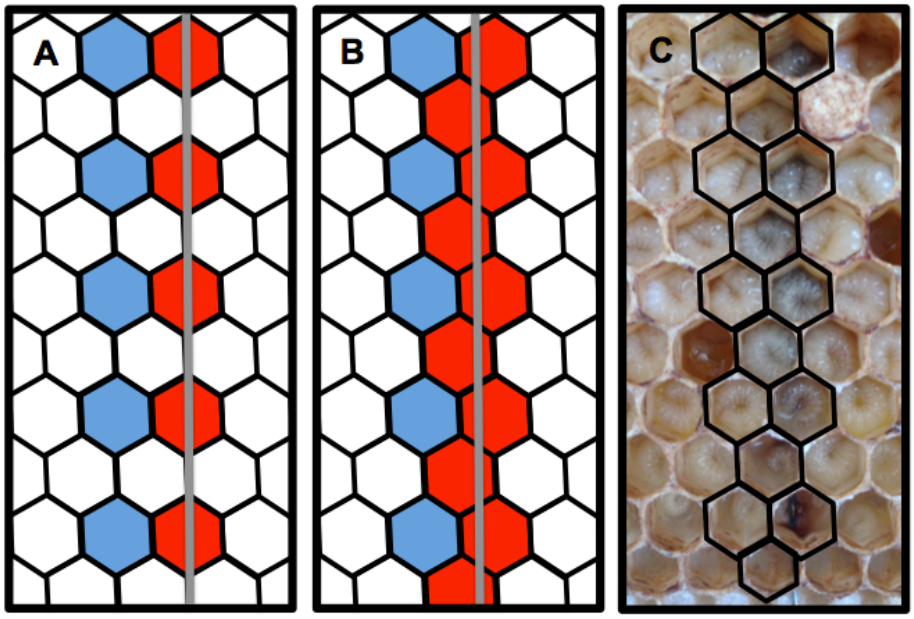

Figure 17. Variations in Wire Alignment with Respect to Brood, Where Wire is either A) Aligned with Cell Center, or B) Unaligned with Cell Center. Red indicates cells overlapping the wire, blue indicates cells adjacent to the wire. Image C. demonstrates a wire unaligned with cell center (outlined cells on the right and in the middle), and the consequent discoloration of affected brood.

CHAPTER V

DISCUSSION

Addressing three central hypotheses, this dissertation explores the relationships between honey bee stressors, brood signals, hygienic behavior and selective breeding. The first hypothesis addressed in this dissertation is that selection for hygienic behavior in honey bees has influenced brood signaling. The second hypothesis is that hygienic behavior and related stressors are associated with chemical brood signals specific to honey bee brood type. Both of these hypotheses predict that hygienic behavior is influenced by colony-type specific brood signals, and evidence provided in Chapters II and III support these hypotheses, as well as this prediction. In Chapter II, brood from hygienic colonies was shown to be more readily removed by all colony types than brood from less hygienic colonies, suggesting that hygienic behavior is influenced by signals originating from the brood. The chemical nature of brood signals associated with hygienic behavior is evidenced in Chapter III by the elevation of P32 in brood infested by mites, brood with high DWV titers, and brood uncapped by nurse bees. With respect to the colony-type specificity of brood signals, Chapter II provides evidence that, despite similarities in the two colony-type's hygiene levels, VSH brood was more likely to be removed than HYG brood in 2014. In Chapter III, the stressor eliciting P32 response was shown to differ between brood types, further supporting the notion that brood signals are colony-type specific, at least in terms of the stressors that trigger them. The third

hypothesis addressed in this dissertation is that the steel wires commonly used to add stability to wax comb foundation negatively affect honey bee brood health. This hypothesis is associated with the predictions that brood in cells overlapping wires 1) incorporate metals from the wires into their tissue and 2) are removed at a higher rate than brood in cells not overlapping wires. Support for this hypothesis is provided in Chapter IV, which presents evidence of higher iron content and higher removal rates of brood in cells overlapping steel foundation wires.

Previous findings upon which the Chapter II and III study designs were based are largely consistent with data presented in Chapter II. For example, the majority of relative and absolute removal rates by various colony-types (Danka et al., 2013; Harbo & Harris, 2005; Ibrahim & Spivak, 2006; Spivak & Reuter, 2001b; Toufailia et al., 2014) and of brood exposed to various honey bee stressors (Harris et al., 2010; Spivak, 1996) are similar to those reported in the literature. However high removal rates of CON brood and low removal rates by HYG colonies in 2013 were unexpected. For CON brood, unexpected results were driven by a single colony, which had higher removal rates than either of the other two CON colonies tested in 2013. In contrast, removal by HYG colonies was consistently low across all four HYG colonies tested in 2013. The reason for this is unclear, although the breeding programs from which the HYG and CON queens were sourced are not as strictly controlled as the breeding program from which VSH queens were sourced. Background data presented in Chapter III was also largely consistent with that from previous studies. Each of the alkanes, alkenes and methylalkanes identified on the cuticles of honey bees and reported in Chapter III have

been identified as constituents of honey bee cuticles in multiple previous studies (Baracchi et al., 2012; Ferreira-Caliman et al., 2012; Piccolo et al., 2010; Richard et al., 2008; Salvy et al., 2001; Schmitt et al., 2007). Compound identification was determined using two mass spectral libraries and verified using an external standard composed of even-numbered alkanes from C8 to C40, confirming the reliability and independence of the honey bee chemical profiles reported.

Many of the novel findings presented in Chapters II and III are also predicted by the literature. The finding that hygienic behavior is influenced by signals originating from the brood is predicted by high removal rates of *Varroa*-infested brood compared to controls (Harris et al., 2010; Spivak, 1996), together with evidence that hygienic behavior is not affected by *Varroa* movement (Aumeier & Rosenkranz, 2001), scent (Aumeier & Rosenkranz, 2001; Le Conte et al., 2015), or offspring (Harris et al., 2010). The concept of honey bee brood signaling is also predicted by previous studies that indicate that cuticular hydrocarbons, commonly used for communication by insects (Blomquist & Vogt, 2003; Howard, 1993; Howard & Blomquist, 2005), are altered in mite-infested (Annoscia et al., 2012; Salvy et al., 2001) and DWV-infected (Schöning et al., 2012) brood. In addition, studies have reported genetic and/or proteomic discrepancies between hygienic and non-hygienic brood (Boutin et al., 2015; Le Conte et al., 2011; Navajas et al., 2008; Parker et al., 2012; Tsuruda et al., 2012), supporting evidence presented in Chapters II and III that hygienic brood is capable of signaling stress. The influence of mites and DWV on P32 quantities presented in Chapter III are consistent with previous studies that have linked honey bee stressors to an increase in high molecular weight

molecules in adults (Baracchi et al., 2012), and to P32 specifically in both brood (Nazzi et al., 2002; Salvy et al., 2001) and adults (Richard et al., 2008). The apparent influence of P32 on behavior is further supported by the notion that bent compounds, including alkenes, are more easily detected than linear alkanes (Dani et al., 2001), as well as evidence of the influence of alkenes on antagonistic behaviors in honey bees and other social insects (Dani et al., 2001; Nascimento & Nascimento, 2012). In addition, the low volatility of P32 is likely conducive to distinguishing diseased and healthy brood, as it allows cell-specific localization of diseased brood odor where high volatility might make such precision difficult.

Recent findings that genes involved in metabolism are up-regulated in VSH bee antennae (Mondet et al., 2015) indicate that after signal perception, VSH bees may be able to rapidly restore sensory thresholds, and thus receiver sensitivity to odors. This suggests that there may be changes in the signal receiver that complement the ability of brood to produce signals. This possibility is also supported by a recent study that reported the over-expression of hydrolase activity related to cytochrome P450 pathways in the brains of non-hygienic bees (Boutin et al., 2015). Since P450s are involved in pheromone decomposition (Feyereisen, 1999), overexpression of cytochrome P450s in non-hygienic honey bees may lead to the rapid breakdown of chemical triggers for hygienic behavior (Boutin et al., 2015), such as P32. In other words, hygienic brood and adults may be slower than non-hygienic brood and adults to break down P32, meaning that hygienic brood may maintain P32 signals longer, and that hygienic adults may be slower to

degrade P32 internally, and thus more responsive to P32 when stressed brood is encountered.

Not all previous literature predicts and supports the novel findings presented in Chapters II and III. The lack of elevated P32 quantity as a response to stress in some previous studies (McDonnell et al., 2013; Richard et al., 2012) differs from results presented in Chapter III. However these discrepancies may be a result of variation in chemical extraction methods, honey bee age, colony types used, and/or colony response variability. For example, McDonnell et al. (2013) and Richard et al. (2012) extracted cuticular chemicals using isohexane for five minutes, and pentane for 10 minutes, respectively. In addition, adult bees rather than larvae were analyzed in both studies, and neither study analyzed bees from HYG or VSH colonies. Other inconsistencies between the literature and results presented in Chapters II and III may stem from the fact that much of the previous work regarding honey bee hygienic behavior has focused on adult olfaction. For example, previous studies regarding honey bee hygiene provide evidence of enhanced olfaction of adults that perform hygienic behavior (Spivak et al., 2003) or that are from hygienic colonies (Martin et al., 2002; Masterman et al., 2001; Mondet et al., 2015). Thus, many previous studies would not predict a lack of correlation, much less a negative correlation, between colony hygiene level and brood removal. However previous behavioral studies have not cross-fostered brood between colony types, and thus have not been capable of distinguishing the effects of brood signaling from those of enhanced adult olfaction (Harbo & Harris, 2005; Ibrahim & Spivak, 2006; Spivak, 1996; Spivak & Reuter, 2001b).

While the theories of enhanced brood signaling and enhanced adult olfaction are not mutually exclusive, the idea that brood with high signal intensity would be associated with adults with high olfactory sensitivity is inconsistent with response threshold models, which predict that increasing stimulus strength leads to detection from individuals with lower sensitivity, at least under natural selection (Beshers & Fewell, 2001; Fewell & Page Jr, 1993). However, analysis of the results presented in Chapters II and III in the context of previous studies suggests that augmented hygienic behavior is a consequence of a combination of enhancements in both brood signaling and receiver responsiveness. The positive correlation between the hygiene level of the brood's colony of origin and brood removal and the lack of a correlation between host colony hygiene level and brood removal presented in Chapter II suggest that between brood signaling and receiver responsiveness, the former may be the more influential trait with respect to performance of hygienic behavior.

The significance of these correlations, perhaps with respect to all three treatments, would likely have been strengthened by higher colony-level sample sizes. However high colony level sample sizes can be difficult to achieve in honey bee work, since colonies require both regular maintenance and adequate geographical space for forage. While individual sample sizes within colonies were relatively large for the studies presented in Chapters II and III, the small colony-level sample sizes obtained each year represent a clear limitation of this work, and reveal a common difficulty of working with social insects in general. While data presented in this research was collected over multiple years, another limitation of the findings presented here is the lack of data from the same

colonies over multiple years. The inability to maintain experimental colonies over multiple years prevented analysis of variation in the measured effects over time and for different individuals with the same genetic background. High colony mortality was likely a result of the lack of *Varroa* treatments applied, an effect of the stress associated with frequent colony disturbance during observations and sample collection, and a reflection of high overwintering loss rates (Lee et al., 2015; Spleen et al., 2013; Steinhauer et al., 2015; Steinhauer et al., 2014; vanEngelsdorp et al., 2012) and the general decline of honey bee health (Lee et al., 2015; Nazzi et al., 2012; Potts et al., 2010). Results presented in Chapter III are also limited by the use of only the non-polar solvent hexane for chemical collection. While the chemical associated with honey bee stressors in this study is non-polar, the possibility that one or more polar chemicals may have a similar effect cannot be excluded. Despite these limitations, treatment effects on removal were consistent within and between years in the cross-fostering experiments (Chapter II). Similarly, treatment and DWV effects on P32 levels were consistent within and between years in the chemical experiments, and cell type effects on removal were consistent between years in the uncapping experiments. Furthermore, similarity between virus levels of capped and uncapped samples in 2014 and in 2015 suggests that DWV was not related to uncapping of VSH brood, and that elevated P32 in uncapped VSH brood was not a result of higher DWV titers in those individuals (Experiment II, Chapter III). This is consistent with evidence that the *Varroa* mite (rather than DWV) triggers elevated P32 in VSH brood (Experiment I, Chapter III). Despite using different colonies in different years as well as some other inconsistencies, consistencies with respect to the effects of

treatment and DWV on P32 levels reveal the robustness of the findings reported in this dissertation, and suggest that the implications of this work are applicable on a broad scale.

Like many of the results presented in Chapters II and III, results presented in Chapter IV regarding honey bee chemical contamination and removal behavior associated with steel wires are consistent with expectations from previous studies. While these experiments were the first to associate brood from cells overlapping steel wires with increased iron content, the findings are consistent with evidence from a previous study associating steel wires with increased iron content of honey (Özcan & Juhaimi, 2012). Similarly, the association of steel wires with increased brood removal rate presented in Chapter IV is consistent with observations of a lack of brood in cells along steel wires (Delaplane, 2006), and with evidence of the toxicity of metals to honey bees (Hladun et al., 2013; Hladun et al., 2012; Nikolić et al., 2015; Søvik et al., 2015). According to results presented in Chapter IV, previous reference to empty cells along wires being a result of the failure of queens to lay eggs in those cells (Delaplane, 2006) may have been an incorrect assumption, as no evidence was provided to support a reluctance to lay rather than hygienic brood removal.

As with Chapters II and III, small colony-level sample sizes obtained for both the chemical and behavioral wire experiments presented in Chapter IV represent a limitation of this work. Small individual samples sizes for 2014 chemical analysis (limited by funding) were likely responsible for the inconclusive results regarding brood iron content in 2014. However, the suggestive nature of these results led to a repeat of the study with a larger sample size in 2015. Other limitations of this work include evidence for variation

in metal composition between years, and the fact that all wire-stabilized frames used in Chapter IV experiments were newly drawn out, and thus longevity of measured effects could not be determined. Although chemical analysis of samples was limited to only 10 elements, the elements chosen for analysis were based on previous studies of elements leeching from steel (Krachler & Shotyk, 2009), and thus represent the elements mostly likely to leech from steel wires into honey bee brood. Nevertheless, it is possible that elements not measured in this study played a role in the increased removal rates observed. Despite limitations of this study, wire effects on brood iron content and removal were consistent between years. Additionally, wire effects on brood removal were consistent between colonies tested within the same year. These consistencies reveal the robustness of our results, and suggest that the implications of this work are applicable on a broad scale.

The broad-scale applicability of results presented in Chapters II, III and IV is especially meaningful in the light of the potential these findings have to make positive impacts on honey bee health. The high potential of these results to be beneficial to honey bees stems primarily from the practicality of the solutions they imply. For example, selective breeding for enhanced hygiene is not a novel idea; rather it is a well-tested and relatively successful practice that has been clearly demonstrated to be beneficial to honey bee health. A transition from the use of liquid nitrogen to the use of a P32-based spray for selective breeding of enhanced hygiene would represent not only an improvement on selection through the mite and DWV-specificity of the selective agent, but also increased practicality for beekeepers, many of whom do not have ready access to liquid nitrogen.

Similarly, a transition away from steel wire stabilizers would represent a simple and practical solution to what is clearly a harmful practice common to modern beekeeping.

However, before extensive actions are taken based on lessons learned from these studies, a few additional studies should be conducted. For example, before development of colony treatments and selective breeding assays to follow up on results from Chapters II and III, steps should be taken to characterize P32. Specifically, the exact location of the double bond in the active isomer should be determined. While the primary isomer of tritriacontene in honey bees contains a double bond on the 10^{th} carbon (Baracchi et al., 2012; Ferreira-Caliman et al., 2012; Salvy et al., 2001), isomers containing double bonds on the 8^{th}, 9^{th}, 12^{th}, and 14^{th} carbons have also been identified (Ferreira-Caliman et al., 2012; Salvy et al., 2001). Additionally, it is important to note that all evidence for a link between P32 and hygienic behavior presented in Chapter III is correlative. An important next step in revealing the nature of the relationship between this apparent signal and response is to test for a causative relationship between P32 and hygienic behavior. Only after these studies are complete should researchers move towards development of P32-based techniques to improve the control of honey bee parasites and diseases. During development of these techniques, it will be important to establish the effective absolute quantity of P32 associated with initiation of hygienic behavior, as quantitative P32 data presented here is not absolute, but relative to the quantities of other chemicals measured in the cuticular profiles of honey bee brood.

With respect to follow up of findings from Chapter IV, additional studies should be conducted to determine how time and variation in metal composition affect chemical

contamination and removal of wire-associated brood. If the negative effects of steel wire stabilizers decrease significantly over time, it may be worth exploring the relative risks of using old comb (Evans & Spivak, 2010) versus using new wire stabilizers. Additionally, new types of stabilizers including wood, plastic, and other compositions of metal should be tested as potential alternatives to the steel wire currently used for stabilization of wax comb. Follow-up studies might also explore the trigger for hygienic removal of wire-contaminated brood. Though results are not reported here, no difference in relative P32 quantity was found in a preliminary investigation of cuticular hydrocarbons of wire-associated and control brood. Exploration of triggers for removal of brood exposed to abiotic stressors such as steel wire would be useful for providing a broader understanding of honey bee hygiene.

In trying to do good for themselves and their bees, beekeepers often end up doing harm to their bees, and thus themselves. For example, in trying to reduce parasite and disease loads by applying miticides to their colonies, beekeepers unintentionally inflict lethal and sublethal damage to their bees. The results presented in Chapters II and III address known challenges to honey bee health, *Varroa* and DWV, and provide tools for the development of sustainable alternatives to the use of harmful miticides. While use of miticides in highly infested colonies may do more good than harm, evidence presented in Chapters II and III suggests that techniques for improving biological control of *Varroa* may be within reach. Improved biological control of *Varroa* may make it possible to avoid miticide use altogether, and thus avoid the negative consequences it has on honey bee, beekeeper, and environmental health. Results presented in Chapter IV address a

previously unresolved challenge to honey bee health: the harm caused by steel wires commonly used in wax comb foundation. In trying to improve the stability of wax combs, beekeepers unknowingly reduce colony populations, likely reducing overwintering success within their own apiaries as a result. With lessons learned from evidence presented in Chapter IV, beekeepers can reduce or avoid use of wax comb stabilizers that are likely to harm their bees.

The honey bee colony is a complex, intriguing, and valuable entity important to science, agriculture and natural ecosystems alike. The lessons learned from this dissertation's exploration of honey bee stressors, brood signals, and hygienic behavior have the potential to improve honey bee health both in the short term, by discouraging the use of steel wire stabilizers in wax comb, and in the long term, by providing the tools needed for the development of sustainable, biological strategies to combat the *Varroa* mite and the pathogens it vectors. This work, and that which will build on it, are of utmost importance in light of the severity of recent honey bee health threats, and the ever-growing demand for crop pollinators. Furthermore the knowledge gained from this research may lead to important insights regarding intraspecific communication in other social insects, including both beneficial and pest species. Honey bees have peaked human curiosity for millennia, all the while providing us with an abundance of goods, services, amusement, and biological insight. No doubt, a multitude of valuable lessons still await within the confines of "the nice symmetry of these small cells, where on each angle genuine science dwells" (Carr, 1880).

References

Aizen MA, Garibaldi LA, Cunningham SA & Klein AM (2008) Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. Current biology 18: 1572-1575.

Aizen MA & Harder LD (2009) The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Current biology 19: 915-918.

Amdam GV, Hartfelder K, Norberg K, Hagen A & Omholt SW (2004) Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite *Varroa destructor* (Acari: Varroidae): a factor in colony loss during overwintering? Journal of economic entomology 97: 741-747.

Anderson D & Trueman J (2000) *Varroa jacobsoni* (Acari: Varroidae) is more than one species. Experimental & Applied Acarology 24: 165-189.

Andersson M (1984) The Evolution of Eusociality. Annual Review of Ecology and Systematics: 165-189.

Annoscia D, Del Piccolo F & Nazzi F (2012) How does the mite *Varroa destructor* kill the honeybee *Apis mellifera*? Alteration of cuticular hydrcarbons and water loss in infested honeybees. Journal of Insect Physiology 58: 1548-1555.

Arathi H, Burns I & Spivak M (2000) Ethology of hygienic behaviour in the honey bee *Apis mellifera* L.(Hymenoptera: Apidae): behavioural repertoire of hygienic bees. Ethology 106: 365-379.

Aumeier P & Rosenkranz P (2001) Scent or movement of *Varroa destructor* mites does not elicit hygienic behaviour by Africanized and Carniolan honey bees. Apidologie 32: 253-264.

Aumeier P, Rosenkranz P & Francke W (2002) Cuticular volatiles, attractivity of worker larvae and invasion of brood cells by *Varroa* mites. A comparison of Africanized and European honey bees. Chemoecology 12: 65-75.

Ayasse M, Engels W, Lübke G, Taghizadeh T & Francke W (1999) Mating expenditures reduced via female sex pheromone modulation in the

primitively eusocial halictine bee, *Lasioglossum (Evylaeus) malachurum* (Hymenoptera: Halictidae). Behavioral Ecology and Sociobiology 45: 95-106.

Bailey L & Ball B (1991) Honey Bee Pathology. Academic Press, London.

Baracchi D, Fadda A & Turillazzi S (2012) Evidence for antiseptic behaviour towards sick adult bees in honey bee colonies. Journal of Insect Physiology 58: 1589-1596.

Bertholf L (1925) The moults of the honeybee. Journal of economic entomology 18: 380-384.

Beshers SN & Fewell JH (2001) Models of division of labor in social insects. Annual review of entomology 46: 413-440.

Biesmeijer JC, Roberts S, Reemer M, Ohlemüller R, Edwards M, Peeters T, Schaffers A, Potts S, Kleukers R & Thomas C (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313: 351-354.

Blomquist GJ & Vogt RG (2003) Insect pheromone biochemistry and molecular biology: The biosynthesis and detection of pheromones and plant volatiles. Elsevier Academic Press, San Diego, CA.

Boecking O, Bienefeld K & Drescher W (2000) Heritability of the Varroa‐specific hygienic behaviour in honey bees (Hymenoptera: Apidae). Journal of Animal Breeding and genetics 117: 417-424.

Boecking O & Drescher W (1992) The removal response of *Apis mellifera* L. colonies to brood in wax and plastic cells after artificial and natural infestation with *Varroa jacobsoni* Oud. and to freeze-killed brood. Experimental & Applied Acarology 16: 321-329.

Boecking O & Genersch E (2008) Varroosis–the ongoing crisis in bee keeping. Journal für Verbraucherschutz und Lebensmittelsicherheit 3: 221-228.

Boecking O, Rosenkranz P & Sasaki M (1999) The pore in the hard conical *Apis cerana* drone capping results from a spinning process. Apidologie 30: 513-519.

Boncristiani H, Underwood R, Schwarz R, Evans JD & Pettis J (2012) Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. Journal of Insect Physiology 58: 613-620.

Bourke AF (1995) Social Evolution in Ants. Princeton University Press.

Boutin S, Alburaki M, Mercier P-L, Giovenazzo P & Derome N (2015) Differential gene expression between hygienic and non-hygienic honeybee (Apis mellifera L.) hives. BMC genomics 16: 500.

Bowen-Walker P, Martin S & Gunn A (1999) The Transmission of Deformed Wing Virus between Honeybees (*Apis mellifera* L.) by the Ectoparasitic Mite *Varroa jacobsoni* Oud. Journal of invertebrate pathology 73: 101-106.

Bozic J & Valentincic T (1995) Quantitative analysis of social grooming behavior of the honey bee *Apis mellifera carnica*. Apidologie 26: 141-141.

Brian M (1975) Larval recognition by workers of the ant Myrmica. Animal Behaviour 23: 745-756.

Brodschneider R & Crailsheim K (2010) Nutrition and health in honey bees. Apidologie 41: 278-294.

Bromenshenk J, Gudatis J, Carlson S, Thomas J & Simmons M (1991) Population dynamics of honey bee nucleus colonies exposed to industrial pollutants. Apidologie 22: 359-369.

Bujok B, Kleinhenz M, Fuchs S & Tautz J (2002) Hot spots in the bee hive. Naturwissenschaften 89: 299-301.

Burley LM, Fell RD & Saacke RG (2008) Survival of honey bee (Hymenoptera: Apidae) spermatozoa incubated at room temperature from drones exposed to miticides. Journal of economic entomology 101: 1081-1087.

Carr W (1880) Introduction or Early History of Bees and Honey. J. Roberts Printer, Salford.

Carreck NL (2011) Varroa. Still a problem in the 21st century? International Bee Research Association.

Chen Y, Pettis JS, Evans JD, Kramer M & Feldlaufer MF (2004) Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. Apidologie 35: 441-448.

Chen YP & Siede R (2007) Honey bee viruses. Advances in virus research 70: 33-80.

Choe D-H, Millar JG & Rust MK (2009) Chemical signals associated with life inhibit necrophoresis in Argentine ants. Proceedings of the National Academy of Sciences 106: 8251-8255.

Chouvenc T, Robert A, Sémon E & Bordereau C (2012) Burial behaviour by dealates of the termite *Pseudacanthotermes spiniger* (Termitidae, Macrotermitinae) induced by chemical signals from termite corpses. Insectes Sociaux 59: 119-125.

Claudianos C, Ranson H, Johnson R, Biswas S, Schuler M, Berenbaum M, Feyereisen R & Oakeshott J (2006) A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect molecular biology 15: 615-636.

Collins AM, Pettis JS, Wilbanks R & Feldlaufer MF (2004) Performance of honey bee (Apis mellifera) queens reared in beeswax cells impregnated with coumaphos. Journal of Apicultural Research 43: 128-134.

Council NR (2006) Status of Pollinators in North America: National Academies Press, Committee on the Status of Pollinators in North America., USA.

Cremer S, Armitage SAO & Schmid-Hempel P (2007) Social immunity. Current Biology 17: R693-R702.

Crozier RH (1970) Coefficients of relationship and the identity of genes by descent in the Hymenoptera. American Naturalist: 216-217.

D'Aubeterre JP, Myrold DD, Royce LA & Rossignol PA (1999) A scientific note of an application of isotope ratio mass spectrometry to feeding by the mite, *Varroa jacobsoni* Oudemans, on the honeybee, *Apis mellifera* L. Apidologie 30: 351-352.

Dade HA (2009) Anatomy and dissection of the honey bee Revised Edition edn. International Bee Research Association, Cardiff, UK.

Dani FR, Jones GR, Destri S, Spencer SH & Turillazzi S (2001) Deciphering the recognition signature within the cuticular chemical profile of paper wasps. Animal Behaviour 62: 165-171.

Danka RG, Harris JW, Villa JD & Dodds GE (2013) Varying congruence of hygienic responses to *Varroa destructor* and freeze-killed brood among different types of honeybees. Apidologie 44: 447-457.

de Guzman LI & Rinderer TE (1999) Identification and comparison of Varroa species infesting honey bees. Apidologie 30: 85-95.

De Jong D, De Jong P & Goncalves L (1982) Weight loss and other damage to developing worker honeybees from infestation with *Varroa jacobsoni*. Journal of apicultural research 21: 165-167.

De Miranda JR & Genersch E (2010) Deformed Wing Virus. Journal of invertebrate pathology 103: S48-S61.

Dekeyser MA & Downer RGH (1994) Biochemical and physiological targets for miticides. Pesticide science 40: 85-101.

Delaplane KS (2006) Honey Bees and Beekeeping: A Year in the Life of an Apiary. Third Edition edn. The University of Georgia, Center for Continuing Education, Georgia, USA.

Delaplane KS, Berry JA, Skinner JA, Parkman JP & Hood WM (2005) Integrated pest management against *Varroa destructor* reduces colony mite levels and delays treatment threshold. Journal of apicultural research 44: 157-162.

Delaplane KS, van der Steen J & Guzman-Novoa E (2013) Standard methods for estimating strength parameters of *Apis mellifera* colonies. Journal of apicultural research 52: 1-12.

Di Pasquale G, Salignon M, Le Conte Y, Belzunces LP, Decourtye A, Kretzschmar A, Suchail S, Brunet J-L & Alaux C (2013) Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? PloS one 8: e72016.

Dietemann V, Nazzi F, Martin SJ, Anderson DL, Locke B, Delaplane KS, Wauquiez Q, Tannahill C, Frey E & Ziegelmann B (2013) Standard methods for varroa research. Journal of Apicultural Research 52: 1.

Dietemann V, Pflugfelder J, Anderson D, Charrière J-D, Chejanovsky N, Dainat B, De Miranda JR, Delaplane K, Dillier F-X & Fuch S (2012) *Varroa destructor*: research avenues towards sustainable control. Journal of apicultural research 51: 125-132.

Ellis J (2012) The honey bee crisis. Outlooks on Pest Management 23: 35-40.

Endler JA (1993) Some general comments on the evolution and design of animal communication systems. Philosophical Transactions of the Royal Society B: Biological Sciences 340: 215-225.

Espinosa-Montano LG-N, E; Sanchez-Albarrant, A; Montaldo, H.; Correa-Benitex, A. (2008) Comparative study of three assays to evaluate hygienic behavior in honey bee (Apis meiiifera L.) colonies. Veterinaria Mexico 39: 39-53.

Evans J, Aronstein K, Chen Y, Hetru C, Imler JL, Jiang H, Kanost M, Thompson G, Zou Z & Hultmark D (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect molecular biology 15: 645-656.

Evans JD, Donovall L, Mullin C, Frazier M, Frazier J, Tarpy DR, Hayes J & Pettis JS (2009) "Entombed Pollen": A new condition in honey bee colonies associated with increased risk of colony mortality. Journal of invertebrate pathology 101: 147-149.

Evans JD & Schwarz RS (2011) Bees brought to their knees: microbes affecting honey bee health. Trends in microbiology 19: 614-620.

Evans JD & Spivak M (2010) Socialized medicine: individual and communal disease barriers in honey bees. Journal of invertebrate pathology 103: S62-S72.

Fakhimzadeh K (2001) Effectiveness of confectioner sugar dusting to knock down Varroa destructor from adult honey bees in laboratory trials. Apidologie 32: 139-148.

Farooqui T (2008) Iron-induced oxidative stress modulates olfactory learning and memory in honeybees. Behavioral neuroscience 122: 433.

Ferreira-Caliman M, Turatti I, Lopes N, Zucchi R & Nascimento F (2012) Analysis of insect cuticular compounds by non-lethal solid phase micro extraction with styrene-divinylbenzene copolymers. Journal of chemical ecology 38: 418-426.

Fewell J & Page Jr R (1993) Genotypic variation in foraging responses to environmental stimuli by honey bees, *Apis mellifera*. Experientia 49: 1106-1112.

Feyereisen R (1999) Insect P450 enzymes. Annual review of entomology 44: 507-533.

Foley K, Fazio G, Jensen AB & Hughes WO (2012) Nutritional limitation and resistance to opportunistic *Aspergillus* parasites in honey bee larvae. Journal of invertebrate pathology 111: 68-73.

Francis B, Blanton W, Littlefield J & Nunamaker R (1989) Hydrocarbons of the cuticle and hemolymph of the adult honey bee (Hymenoptera: Apidae). Annals of the Entomological Society of America 82: 486-494.

Francis B, Blanton W & Nunamaker R (1985) Extractable surface hydrocarbons of workers and drones of the genus Apis. Journal of apicultural research 24: 13-26.

Frazier M, Mullin C, Frazier J & Ashcraft S (2008) What have pesticides got to do with it? American Bee Journal 148: 521-524.

Free J & Spencer‐Booth Y (1959) The longevity of worker honey bees (*Apis mellifera*), Vol. 34: Proceedings of the Royal Entomological Society of London. Series A, General Entomology (ed. Wiley Online Library, pp. 141-150.

Free J & Winder M (1983) Brood recognition by honeybee (*Apis mellifera*) workers. Animal Behaviour 31: 539-545.

Frey E, Odemer R, Blum T & Rosenkranz P (2013) Activation and interruption of the reproduction of Varroa destructor is triggered by host signals (Apis mellifera). Journal of Invertebrate Pathology.

Frisch Kv (1950) Bees: Their Vision, Chemical Senses, and Language. Cornell University Press, Ithaca, NY.

Fukuda H & Sakagami SF (1968) Worker brood survival in honeybees. Researches on Population Ecology 10: 31-39.

Garedew A, Schmolz E & Lamprecht I (2004) The energy and nutritional demand of the parasitic life of the mite *Varroa destructor*. Apidologie 35: 419-430.

Genersch E, Von Der Ohe W, Kaatz H, Schroeder A, Otten C, Büchler R, Berg S, Ritter W, Mühlen W & Gisder S (2010) The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41: 332-352.

Gilliam M, Taber III S & Richardson GV (1983) Hygienic behavior of honey bees in relation to chalkbrood disease. Apidologie 14: 29-39.

Gilliam M, Taber S, Lorenz BJ & Prest DB (1988) Factors affecting development of chalkbrood disease in colonies of honey bees, *Apis mellifera*, fed pollen

contaminated with *Ascosphaera apis*. Journal of invertebrate pathology 52: 314-325.

Ginzel MD, Blomquist GJ, Millar JG & Hanks LM (2003) Role of contact pheromones in mate recognition in *Xylotrechus colonus*. Journal of chemical ecology 29: 533-545.

Gisder S, Aumeier P & Genersch E (2009) Deformed wing virus: replication and viral load in mites (*Varroa destructor*). Journal of General Virology 90: 463-467.

Glancey BM, Stringer C, Craig C, Bishop P & Martin B (1970) Pheromone may induce brood tending in the fire ant, Solenopsis saevissima. Nature Vol. 226 pp. 863-864 pp.

Goode K, Huber Z, Mesce KA & Spivak M (2006) Hygienic behavior of the honey bee (*Apis mellifera*) is independent of sucrose responsiveness and foraging ontogeny. Hormones and behavior 49: 391-397.

Gramacho KP & Spivak M (2003) Differences in olfactory sensitivity and behavioral responses among honey bees bred for hygienic behavior. Behavioral Ecology and Sociobiology 54: 472-479.

Grixti JC, Wong LT, Cameron SA & Favret C (2009) Decline of bumble bees (Bombus) in the North American Midwest. Biological Conservation 142: 75-84.

Gullan PJ & Cranston PS (2010) The Insects: An Outline of Entomology. 4th Edition edn. John Wiley & Sons, West Sussex, UK.

Haarmann T, Spivak M, Weaver D, Weaver B & Glenn T (2002) Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. Journal of economic entomology 95: 28-35.

Hamilton WD (1964) The genetical evolution of social behaviour. . Journal of theoretical biology 7: 17-52.

Harbo J (1979) The rate of depletion of spermatozoa in the queen honeybee spermatheca. J. Apic. Res 18: 204-207.

Harbo JR (1986) Effect of population size on brood production, worker survival and honey gain in colonies of honeybees. Journal of apicultural research 25: 22-29.

Harbo JR & Harris JW (2001) Resistance to Varroa destructor (Mesostigmata: Varroidae) when mite-resistant queen honey bees (Hymenoptera: Apidae) were free-mated with unselected drones. Journal of economic entomology 94: 1319-1323.

Harbo JR & Harris JW (2005) Suppressed mite reproduction explained by the behaviour of adult bees. Journal of apicultural research 44: 21-23.

Harbo JR & Harris JW (2009) Responses to Varroa by honey bees with different levels of Varroa Sensitive Hygiene. Journal of apicultural research 48: 156.

Harris JW (2007) Bees with Varroa Sensitive Hygiene preferentially remove mite infested pupae aged ≤ five days post capping. J Apic Res 46: 134-139.

Harris JW (2008) Effect of brood type on *Varroa*-sensitive hygiene by worker honey bees (Hymenoptera: Apidae). Annals of the Entomological Society of America 101: 1137-1144.

Harris JW, Danka RG & Villa JD (2010) Honey bees (Hymenoptera: Apidae) with the trait of varroa sensitive hygiene remove brood with all reproductive stages of varroa mites (Mesostigmata: Varroidae). Annals of the Entomological Society of America 103: 146-152.

Harris JW, Danka RG & Villa JD (2012) Changes in infestation, cell cap condition, and reproductive status of *Varroa destructor* (Mesostigmata: Varroidae) in brood exposed to honey bees with Varroa sensitive hygiene. Annals of the Entomological Society of America 105: 512-518.

Hayworth MK, Johnson NG, Wilhelm ME, Gove RP, Metheny JD & Rueppell O (2009) Added weights lead to reduced flight behavior and mating success in polyandrous honey bee queens (*Apis mellifera*). Ethology 115: 698-706.

Henry M, Beguin M, Requier F, Rollin O, Odoux J-F, Aupinel P, Aptel J, Tchamitchian S & Decourtye A (2012) A common pesticide decreases foraging success and survival in honey bees. Science 336: 348-350.

Herrmann M, Kanbar G & Engels W (2005) Survival of honey bee (*Apis mellifera*) pupae after trypan blue staining of wounds caused by *Varroa destructor* mites or artificial perforation. Apidologie 36: 107-111.

Highfield AC, El Nagar A, Mackinder LC, Laure M-LN, Hall MJ, Martin SJ & Schroeder DC (2009) Deformed wing virus implicated in overwintering honeybee colony losses. Applied and environmental microbiology 75: 7212-7220.

Hladun KR, Kaftanoglu O, Parker DR, Tran KD & Trumble JT (2013) Effects of selenium on development, survival, and accumulation in the honeybee (*Apis mellifera* L.). Environmental Toxicology and Chemistry 32: 2584-2592.

Hladun KR, Smith BH, Mustard JA, Morton RR & Trumble JT (2012) Selenium toxicity to honey bee (*Apis mellifera* L.) pollinators: effects on behaviors and survival. PloS one 7: 1-10.

Holden C (2006) Report warns of looming pollination crisis in North America. Science 314: 397-397.

Hooven L, Sagili R & Johansen E (2013) How to Reduce Bee Poisoning from Pesticides: Oregon State University Extension Service.

Hoover SER, Keeling CI, Winston ML & Slessor KN (2003) The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften 90: 477-480.

Howard RW (1993) Cuticular hydrocarbons and chemical communication. University of Nebraska Press, Lincoln, NE.

Howard RW & Blomquist GJ (1982) Chemical ecology and biochemistry of insect hydrocarbons. Annual review of entomology 27: 149-172.

Howard RW & Blomquist GJ (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons Annu. Rev. Entomol. 50: 371-393.

Huang Z-Y & Robinson GE (1992) Honeybee colony integration: worker-worker interactions mediate hormonally regulated plasticity in division of labor. Proceedings of the National Academy of Sciences 89: 11726-11729.

Huang Z-Y & Robinson GE (1996) Regulation of honey bee division of labor by colony age demography. Behavioral Ecology and Sociobiology 39: 147-158.

Hughes WOH, Oldroyd BP, Beekman M & Ratnieks FL (2008) Ancestral monogamy shows kin selection is key to the evolution of eusociality. Science 320: 1213-1216.

Ibrahim A, Reuter GS & Spivak M (2007) Field trial of honey bee colonies bred for mechanisms of resistance against *Varroa destructor*. Apidologie 38: 67-76.

Ibrahim A & Spivak M (2006) The relationship between hygienic behavior and suppression of mite reproduction as honey bee (*Apis mellifera*) mechanisms of resistance to *Varroa destructor*. Apidologie 37: 31.

Ifantidis MD (1988) Some aspects of the process of *Varroa jacobsoni* mite entrance into honey bee (*Apis mellifera*) brood cells. Apidologie 19: 387-396.

Imdorf A, Charrière J-D, Kilchenmann V, Bogdanov S & Fluri P (2003) Alternative strategy in central Europe for the control of *Varroa destructor* in honey bee colonies. Apiacta 38: 258-285.

Ioannidou M, Zachariadis G, Anthemidis A & Stratis J (2005) Direct determination of toxic trace metals in honey and sugars using inductively coupled plasma atomic emission spectrometry. Talanta 65: 92-97.

Islam M, Khalil M & Gan SH (2014) Toxic compounds in honey. Journal of Applied Toxicology 34: 733-742.

Jay S (1964) The Cocoon of the Honey Bee, *Apis mellifera* L. The Canadian Entomologist 96: 784-792.

Jimenez-Del-Rio M, Guzman-Martinez C & Velez-Pardo C (2010) The effects of polyphenols on survival and locomotor activity in *Drosophila melanogaster* exposed to iron and paraquat. Neurochemical research 35: 227-238.

Johansen CA & Kleinschmidt MG (1972) Insecticide formulations and their toxicity to honeybees. Journal of apicultural research 11: 59-62.

Johnson RM, Ellis MD, Mullin CA & Frazier M (2010) Pesticides and honey bee toxicity–USA. Apidologie 41: 312-331.

Johnson RM, Pollock HS & Berenbaum MR (2009) Synergistic interactions between in-hive miticides in *Apis mellifera*. Journal of economic entomology 102: 474-479.

Kanbar G & Engels W (2003) Ultrastructure and bacterial infection of wounds in honey bee (*Apis mellifera*) pupae punctured by Varroa mites. Parasitology research 90: 349-354.

Kapheim KM, Pan H, Li C, Salzberg SL, Puiu D, Magoc T, Robertson HM, Hudson ME, Venkat A & Fischman BJ (2015) Genomic signatures of evolutionary transitions from solitary to group living. Science 348: 1139-1143.

Kather R, Drijfhout FP, Shemilt S & Martin SJ (2015) Evidence for passive chemical camouflage in the parasitic mite *Varroa destructor*. Journal of chemical ecology 41: 178-186.

Kearns CA, Inouye DW & Waser NM (1998) Endangered mutualisms: the conservation of plant-pollinator interactions. Annual Review of Ecology and Systematics: 83-112.

Khoury DS, Myerscough MR & Barron AB (2011) A quantitative model of honey bee colony population dynamics. PloS one 6: e18491.

Kirrane MJ, De Guzman LI, Rinderer TE, Frake AM, Wagnitz J & Whelan PM (2011) Asynchronous development of honey bee host and *Varroa destructor* (Mesostigmata: Varroidae) influences reproductive potential of mites. Journal of economic entomology 104: 1146-1152.

Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C & Tscharntke T (2007) Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences 274: 303-313.

Krachler M & Shotyk W (2009) Trace and ultratrace metals in bottled waters: survey of sources worldwide and comparison with refillable metal bottles. Science of the Total Environment 407: 1089-1096.

Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R & Popov S (1999) Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. Journal of ethnopharmacology 64: 235-240.

Kuster RD, Boncristiani HF & Rueppell O (2014) Immunogene and viral transcript dynamics during parasitic *Varroa destructor* mite infection of developing honey bee (*Apis mellifera*) pupae. The Journal of experimental biology 217: 1710-1718.

Langstroth LL (1914) Langstroth on the Hive and the Honey-bee: A Bee-keeper's Manual. Hopkins, Bridgman & Company.

Le Conte Y, Alaux C, Martin JF, Harbo J, Harris J, Dantec C, Severac D, Cros‐Arteil S & Navajas M (2011) Social immunity in honeybees (*Apis mellifera*): transcriptome analysis of varroa‐hygienic behaviour. Insect molecular biology 20: 399-408.

Le Conte Y, Arnold G, Trouiller J, Masson C & Chappe B (1990) Identification of a brood pheromone in honeybees. Naturwissenschaften 77: 334-336.

Le Conte Y, Huang Z, Roux M, Zeng Z, Christidès J-P & Bagnères A-G (2015) *Varroa destructor* changes its cuticular hydrocarbons to mimic new hosts. Biology Letters 11: 20150233.

Le Conte Y, Sreng L & Poitout SH (1995) Brood Pheromone Can Modulate the Feeding Behavior of Apis mellifera Workers (Hytnenoptera: Apidae). Journal of economic entomology 88: 798-804.

LeConte Y & Hefetz A (2008) Primer pheromones in social hymenoptera. Annu. Rev. Entomol. 53: 523-542.

Lee KV, Steinhauer N, Rennich K, Wilson ME, Tarpy DR, Caron DM, Rose R, Delaplane KS, Baylis K & Lengerich EJ (2015) A national survey of managed honey bee 2013–2014 annual colony losses in the USA. Apidologie 46: 292-305.

Leita L, Muhlbachova G, Cesco S, Barbattini R & Mondini C (1996) Investigation of the use of honey bees and honey bee products to assess heavy metals contamination. Environmental monitoring and assessment 43: 1-9.

Lockey KH (1988) Lipids of the insect cuticle: origin, composition and function. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 89: 595-645.

Losey JE & Vaughan M (2006) The economic value of ecological services provided by insects. Bioscience 56: 311-323.

Martin C, Provost E, Bagnères AG, Roux M, Clément JL & Le Conte Y (2002) Potential mechanism for detection by *Apis mellifera* of the parasitic mite *Varroa destructor* inside sealed brood cells. Physiological Entomology 27: 175-188.

Martin C, Salvy M, Provost E, Bagnères A-G, Roux M, Crauser D, Clement J-L & Le Conte Y (2001) Variations in chemical mimicry by the ectoparasitic mite *Varroa jacobsoni* according to the developmental stage of the host honey-bee *Apis mellifera*. Insect Biochemistry and Molecular Biology 31: 365-379.

Martin SJ (2001) The role of Varroa and viral pathogens in the collapse of honeybee colonies: a modelling approach. Journal of Applied Ecology 38: 1082-1093.

Martin SJ, Highfield AC, Brettell L, Villalobos EM, Budge GE, Powell M, Nikaido S & Schroeder DC (2012) Global Honey Bee Viral Landscape Altered by a Parasitic Mite. Science 336: 1304-1306.

Masterman R, Ross R, Mesce K & Spivak M (2001) Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L.). Journal of Comparative Physiology A 187: 441-452.

Masterman R, Smith B & Spivak M (2000) Brood odor discrimination abilities in hygienic honey bees (*Apis mellifera* L.) using proboscis extension reflex conditioning. Journal of Insect Behavior 13: 87-101.

Maurizio A & Hodges F (1950) The influence of pollen feeding and brood rearing on the length of life and physiological condition of the honeybee preliminary report. Bee World 31: 9-12.

McDonnell CM, Alaux C, Parrinello H, Desvignes J-P, Crauser D, Durbesson E, Beslay D & Le Conte Y (2013) Ecto-and endoparasite induce similar chemical and brain neurogenomic responses in the honey bee (*Apis mellifera*). BMC ecology 13: 25.

Meixner MD (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of invertebrate pathology 103: S80-S95.

Memmott J, Waser NM & Price MV (2004) Tolerance of pollination networks to species extinctions. Proceedings of the Royal Society of London. Series B: Biological Sciences 271: 2605-2611.

Menzel R (2012) The honeybee as a model for understanding the basis of cognition. Nature Reviews Neuroscience 13: 758-768.

Möckel N, Gisder S & Genersch E (2011) Horizontal transmission of deformed wing virus: pathological consequences in adult bees (*Apis mellifera*) depend on the transmission route. Journal of General Virology 92: 370-377.

Mondet F, Alaux C, Severac D, Rohmer M, Mercer AR & Le Conte Y (2015) Antennae hold a key to *Varroa*-sensitive hygiene behaviour in honey bees. Scientific reports 5: 1-12.

Monnin T & Peeters C (1997) Cannibalism of subordinates' eggs in the monogynous queenless ant Dinoponera quadriceps. Naturwissenschaften 84: 499-502.

Morse RA & Calderone NW (2000) The value of honey bees as pollinators of US crops in 2000. Bee Culture 128: 1-15.

Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R & Pettis JS (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PloS one 5: e9754.

Nascimento D & Nascimento F (2012) Acceptance threshold hypothesis is supported by chemical similarity of cuticular hydrocarbons in a stingless bee, *Melipona asilvai*. Journal of chemical ecology 38: 1432-1440.

Nation J, Sanford M & Milne K (1992) Cuticular hydrocarbons from *Varroa jacobsoni*. Experimental & Applied Acarology 16: 331-344.

Naug D (2009) Nutritional stress due to habitat loss may explain recent honeybee colony collapses. Biological Conservation 142: 2369-2372.

Navajas M, Migeon A, Alaux C, Martin-Magniette M-L, Robinson G, Evans J, Cros-Arteil S, Crauser D & Le Conte Y (2008) Differential gene expression of the honey bee *Apis mellifera* associated with *Varroa destructor* infection. BMC genomics 9: 301.

Nazzi F, Brown SP, Annoscia D, Del Piccolo F, Di Prisco G, Varricchio P, Della Vedova G, Cattonaro F, Caprio E & Pennacchio F (2012) Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathogens 8: e1002735.

Nazzi F, Milani N & Della Vedova G (2002) (Z)-8-Heptadecene from infested cells reduces the reproduction of *Varroa destructor* under laboratory conditions. Journal of chemical ecology 28: 2181-2190.

Neumann P & Carreck NL (2010) Honey bee colony losses. Journal of apicultural research 49: 1-6.

Newton D & Ostasiewski N (1986) A Simplified Bioassay for Behavioral Resistance to American Foulbrood in Honey-Bees (*Apis-Mellifera* L). American Bee Journal 126: 278-281.

Nikolić TV, Purać J, Orčić S, Kojić D, Vujanović D, Stanimirović Z, Gržetić I, Ilijević K, Šikoparija B & Blagojević DP (2015) Environmental effects on superoxide dismutase and catalase activity and expression in honey bee. Archives of insect biochemistry and physiology 0: 1-14.

Özcan MM & Juhaimi FYA (2012) Determination of heavy metals in bee honey with connected and not connected metal wires using inductively coupled plasma atomic emission spectrometry (ICP–AES). Environmental monitoring and assessment 184: 2373-2375.

Page RE, Robinson GE, Fondrk MK & Nasr ME (1995) Effects of worker genotypic diversity on honey bee colony development and behavior (*Apis mellifera* L.). Behavioral Ecology and Sociobiology 36: 387-396.

Parker R, Guarna MM, Melathopoulos AP, Moon K-M, White R, Huxter E, Pernal SF & Foster LJ (2012) Correlation of proteome-wide changes with social immunity behaviors provides insight into resistance to the parasitic mite, *Varroa destructor*, in the honey bee (*Apis mellifera*). Genome Biology 13: R81.

Peng Y-SC, Fang Y, Xu S, Ge L & Nasr ME (1987) Response of foster Asian honeybee (*Apis cerana Fabr*.) colonies to the brood of European honeybee (*Apis mellifera L.*) infested with parasitic mite, Varroa jacobsoni Oudemans. Journal of invertebrate pathology 49: 259-264.

Pernal SF, Sewalem A & Melathopoulos AP (2012) Breeding for hygienic behaviour in honeybees (Apis mellifera) using free-mated nucleus colonies. Apidologie: 1-14.

Pettis J, Shimanuki WWH & Teel P (1991) Fluvalinate treatment of queen and worker honey bees (*Apis mellifera L*) and effects on subsequent mortality, queen acceptance and supersedure. Apidologie 22: 1-7.

Pettis JS, Collins AM, Wilbanks R & Feldlaufer MF (2004) Effects of coumaphos on queen rearing in the honey bee, Apis mellifera. Apidologie 35: 605-610.

Pettis JS & Delaplane KS (2010) Coordinated responses to honey bee decline in the USA. Apidologie 41: 256-263.

Piccolo F, Nazzi F, Vedova GD & Milani N (2010) Selection of *Apis mellifera* workers by the parasitic mite *Varroa destructor* using host cuticular hydrocarbons. Parasitology 137: 967-973.

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O & Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. Trends in ecology & evolution 25: 345-353.

Rath W & Drescher W (1990) Response of *Apis cerana* Fabr. towards brood infested with *Varroa jacobsoni* Oud. and infestation rate of colonies in Thailand. Apidologie 21: 311-321.

Richard F, Aubert A & Grozinger C (2008) Modulation of social interactions by immune stimulation in honey bee, *Apis mellifera*, workers. BMC biology 6: 50.

Richard F-J, Holt HL & Grozinger CM (2012) Effects of immunostimulation on social behavior, chemical communication and genome-wide gene expression in honey bee workers (*Apis mellifera*). BMC genomics 13: 558.

Rinderer TE, Harris JW, Hunt GJ & De Guzman LI (2010) Breeding for resistance to *Varroa destructor* in North America. Apidologie 41: 409-424.

Romer F (1991) The oenocytes of insects: differentiation, changes during molting, and their possible involvement in the secretion of molting hormone. AP Gupta edn. Rutgers University Press, New Brunswick, NJ.

Rortais A, Arnold G, Halm M-P & Touffet-Briens F (2005) Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. Apidologie 36: 71-83.

Rosenkranz P, Aumeier P & Ziegelmann B (2010) Biology and control of Varroa destructor. Journal of Invertebrate Pathology 103: S96-S119.

Rosenkranz P & Renz M (2003) *Varroa destructor* infestation of adult bees, worker brood and drone brood during the season and consequences for treatment concepts. Apidologie 34: 509-510.

Rosenkranz P, Tewarson NC, Singh A & Engels W (1993) Differential hygienic behaviour towards *Varroa jacobsoni* in capped worker brood of *Apis cerana* depends on alien scent adhering to the mites. Journal of apicultural research 32: 89-93.

Rucker R, Thurman W & Burgett M (2011) Colony collapse and the economic implications of bee disease.

Rueppell O, Hayworth M & Ross N (2010) Altruistic self‐removal of health‐compromised honey bee workers from their hive. Journal of evolutionary biology 23: 1538-1546.

Rueppell O, Pankiw T, Nielsen DI, Fondrk MK, Beye M & Page RE (2004) The genetic architecture of the behavioral ontogeny of foraging in honeybee workers. Genetics 167: 1767-1779.

Ryabov EV, Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D, Mead A, Burroughs N & Evans DJ (2014) A virulent strain of deformed wing virus (DWV) of honeybees (*Apis mellifera*) prevails after *Varroa destructor*-mediated, or in vitro, transmission. PLoS Pathogens 10: e1004230.

Salvy M, Martin C, Bagneres A, Provost E, Roux M, Le Conte Y & Clement J (2001) Modifications of the cuticular hydrocarbon profile of *Apis mellifera* worker bees in the presence of the ectoparasitic mite *Varroa jacobsoni* in brood cells. Parasitology 122: 145-159.

Sammataro D, Untalan P, Guerrero F & Finley J (2005) The resistance of varroa mites (Acari: Varroidae) to acaricides and the presence of esterase. International journal of acarology 31: 67-74.

Santomauro G, Oldham NJ, Boland W & Engels W (2004) Cannibalism of diploid drone larvae in the honey bee (Apis mellifera) is released by odd pattern of cuticular substances. Journal of apicultural research 43: 69-74.

Schatton-Gadelmayer K & Engel W (1988) Blood proteins and body weight of newly-emerged worker honeybees with different levels of parasitization of brood mites (Hymenoptera: Apidae: *Apis mellifera*/Acarina: Varroidae: *Varroa jacobsoni*). Entomologia generalis 14: 93-101.

Schmickl T & Crailsheim K (2001) Cannibalism and early capping: strategy of honeybee colonies in times of experimental pollen shortages. Journal of Comparative Physiology A 187: 541-547.

Schmidt LS, Schmidt JO, Rao H, Wang W & Xu L (1995) Feeding preference and survival of young worker honey bees (Hymenoptera: Apidae) fed rape, sesame, and sunflower pollen. Journal of economic entomology 88: 1591-1595.

Schmitt T, Herzner G, Weckerle B, Schreier P & Strohm E (2007) Volatiles of foraging honeybees *Apis mellifera* (Hymenoptera: Apidae) and their potential role as semiochemicals. Apidologie 38: 164-170.

Schöning C, Gisder S, Geiselhardt S, Kretschmann I, Bienefeld K, Hilker M & Genersch E (2012) Evidence for damage-dependent hygienic behaviour towards

Varroa destructor-parasitised brood in the western honey bee, *Apis mellifera*. The Journal of experimental biology 215: 264-271.

Shi YY, Huang ZY, Zeng ZJ, Wang ZL, Wu XB & Yan WY (2011) Diet and cell size both affect queen-worker differentiation through DNA methylation in honey bees (Apis mellifera, Apidae). PloS one 6: e18808.

Simone M, Evans JD & Spivak M (2009) Resin collection and social immunity in honey bees. Evolution 63: 3016-3022.

Sokal RR & Rohlf FJ (1995) The principles and practice of statistics in biological research. WH Freeman and Co., New York.

Soroker V & Hefetz A (2000) Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger*. Journal of Insect Physiology 46: 1097-1102.

Søvik E, Perry CJ, LaMora A, Barron AB & Ben-Shahar Y (2015) Negative impact of manganese on honeybee foraging. Biology Letters 11: 20140989.

Spivak M (1996) Honey bee hygienic behavior and defense against *Varroa jacobsoni*. Apidologie 27: 245-260.

Spivak M & Downey DL (1998) Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). Journal of economic entomology 91: 64-70.

Spivak M & Gilliam M (1998) Hygienic behaviour of honey bees and its application for control of brood diseases and Varroa: Part II. Studies on hygienic behaviour since the Rothenbuhler era. Bee World 79: 169-186.

Spivak M, Mader E, Vaughan M & Euliss Jr NH (2011) The Plight of the Bees. Environmental science & technology 45: 34-38.

Spivak M, Masterman R, Ross R & Mesce KA (2003) Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. Journal of neurobiology 55: 341-354.

Spivak M & Reuter GS (2001a) Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. Apidologie 32: 555-565.

Spivak M & Reuter GS (2001b) *Varroa destructor* infestation in untreated honey bee (Hymenoptera: Apidae) colonies selected for hygienic behavior. Journal of economic entomology 94: 326-331.

Spleen AM, Lengerich EJ, Rennich K, Caron D, Rose R, Pettis JS, Henson M, Wilkes JT, Wilson M & Stitzinger J (2013) A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. Journal of apicultural research 52: 44-53.

Spotter A, Gupta P, Nurnberg G, Reinsch N & Bienefeld K (2012) Development of a 44K SNP assay focusing on the analysis of a varroa-specific defence behaviour in honey bees (Apis mellifera carnica). Molecular Ecology Resources.

Spötter A, Gupta P, Nürnberg G, Reinsch N & Bienefeld K (2012) Development of a 44K SNP assay focussing on the analysis of a varroa‐specific defence behaviour in honey bees (*Apis mellifera* carnica). Molecular ecology resources 12: 323-332.

Starks PT, Blackie CA & Seeley TD (2000) Fever in honeybee colonies. Naturwissenschaften 87: 229-231.

Stefanidou M, Athanaselis S & Koutselinis A (2003) The toxicology of honey bee poisoning. Veterinary and human toxicology 45: 261-265.

Steinhauer N, Rennich K, Lee KV, Pettis J, Tarpy DR, Rangel J, Caron D, Sagili R, Skinner JA, Wilson ME, Wilkes JT, Delaplane KS, Rose R & van Engelsdorp D (2015) Colony Loss 2014-2015: Preliminary Results, Vol. 2015.

Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS, Rose R, Skinner JA, Tarpy DR & Wilkes JT (2014) A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. Journal of apicultural research 53: 1-18.

Sun Q & Zhou X (2013) Corpse management in social insects. International journal of biological sciences 9: 313.

Swanson JA, Torto B, Kells SA, Mesce KA, Tumlinson JH & Spivak M (2009) Odorants that induce hygienic behavior in honeybees: identification of volatile compounds in chalkbrood-infected honeybee larvae. Journal of chemical ecology 35: 1108-1116.

Sylvester HA, Watts RP, De Guzman L, Stelzer JA & Rinderer TE (1999) *Varroa* in the mating yard: II. The effects of Varroa and fluvalinate on drone mating competitiveness. American Bee Journal.

Thompson HM (2003) Behavioural effects of pesticides in bees–their potential for use in risk assessment. Ecotoxicology 12: 317-330.

Topolska G, Ball B & Allen M (1995) Identification of viruses in bees from two Warsaw apiaries. Medycyna Weterynaryjna (Poland) 51: 145-147.

Toufailia HMA, Amiri E, Scandian L, Kryger P & Ratnieks FL (2014) Towards integrated control of varroa: effect of variation in hygienic behaviour among honey bee colonies on mite population increase and deformed wing virus incidence. Journal of apicultural research 53: 555-562.

Traynor KS, Le Conte Y & Page RE (2015) Age matters: pheromone profiles of larvae differentially influence foraging behaviour in the honeybee, *Apis mellifera*. Animal Behaviour 99: 1-8.

Tsuruda JM, Harris JW, Bourgeois L, Danka RG & Hunt GJ (2012) High-resolution linkage analyses to identify genes that influence Varroa sensitive hygiene behavior in honey bees. PloS one 7: e48276.

Ulyshen MD & Shelton TG (2012) Evidence of cue synergism in termite corpse response behavior. Naturwissenschaften 99: 89-93.

van der Steen JJ, de Kraker J & Grotenhuis T (2012) Spatial and temporal variation of metal concentrations in adult honeybees (*Apis mellifera* L.). Environmental monitoring and assessment 184: 4119-4126.

Van der Steen JJ, de Kraker J & Grotenhuis T (2015) Assessment of the Potential of Honeybees (*Apis mellifera* L.) in Biomonitoring of Air Pollution by Cadmium, Lead and Vanadium. Journal of Environmental Protection 6: 96.

vanEngelsdorp D, Caron D, Hayes J, Underwood R, Henson M, Rennich K, Spleen A, Andree M, Snyder R & Lee K (2012) A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. Journal of apicultural research 51: 115-124.

vanEnglesdorp D, Hayes Jr J, Underwood RM & Pettis J (2008) A survey of honey bee colony losses in the US, Fall 2007 to Spring 2008. PloS one 3: e4071.

Vaz AH, Blomquist GJ & Reitz RC (1988) Characterization of the fatty acyl elongation reactions involved in hydrocarbon biosynthesis in the housefly, *Musca domestica* L. Insect biochemistry 18: 177-184.

Villa JD, Danka RG & Harris JW (2009) Simplified methods of evaluating colonies for levels of Varroa Sensitive Hygiene (VSH). Journal of apicultural research 48: 162-167.

Villa S, Vighi M, Finizio A & Serini GB (2000) Risk assessment for honeybees from pesticide-exposed pollen. Ecotoxicology 9: 287-297.

Visscher P & Dukas R (1997) Survivorship of foraging honey bees. Insectes Sociaux 44: 1-5.

Waddington KD & Rothenbuhler WC (1976) Behaviour associated with hairless-black syndrome of adult honeybees. Journal of apicultural research 15: 35-41.

Walsh JP & Tschinkel W (1974) Brood recognition by contact pheromone in the red imported fire ant, Solenopsis invicta. Animal Behaviour 22: 695-704.

Wang T-H, Jian C-H, Hsieh Y-K, Wang F-N & Wang C-F (2013) Spatial distributions of inorganic elements in honeybees (*Apis mellifera* L.) and possible relationships to dietary habits and surrounding environmental pollutants. Journal of agricultural and food chemistry 61: 5009-5015.

Watkins J & Cole T (1966) Attraction of army ant workers to secretions of their queens. Texas Journal of Science 18: 254.

Weinstock GM, Robinson GE, Gibbs RA, Worley KC, Evans JD, Maleszka R, Robertson HM, Weaver DB, Beye M & Bork P (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443: 931-949.

Wenner A & Bushing W (1996) *Varroa* mite spread in the United States. Bee culture 124: 341-343.

Wheeler WM (1910) Ants: their structure, development and behavior. Columbia University Press, New York.

Williams I (1994) The dependence of crop production within the European Union on pollination by honey bees. Agricultural Zoology Reviews (United Kingdom) 6: 229-257.

Wilson EO (1965) Chemical communication in the social insects. Science 149: 1064-1071.

Wilson-Rich N, Spivak M, Fefferman NH & Starks PT (2009) Genetic, individual, and group facilitation of disease resistance in insect societies. Annual review of entomology 54: 405-423.

Winston ML (1991) The Biology of the Honey Bee. Harvard University Press, Cambridge, Massachusetts.

Wu JY, Anelli CM & Sheppard WS (2011) Sub-lethal effects of pesticide residues in brood comb on worker honey bee (*Apis mellifera*) development and longevity. PloS one 6: e14720.

Yang X & Cox-Foster D (2007) Effects of parasitization by *Varroa destructor* on survivorship and physiological traits of *Apis mellifera* in correlation with viral incidence and microbial challenge. Parasitology 134: 405-412.

Yang X & Cox-Foster DL (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proceedings of the National Academy of Sciences of the United States of America 102: 7470-7475.

Zhu W, Schmehl DR, Mullin CA & Frazier JL (2014) Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. PloS one 9: e77547.